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(54) Title: SCREENING METHODS USING G-PROTEIN COUPLED RECEPTORS AND RELATED COMPOSITIONS

(57) Abstract: The present invention provides screening methods for GPCRs based on the discovery that the affinity of a receptor agonist for a GPCR (such as the parathyroid hormone receptor) when not bound to a G-protein is correlated with the length of time over which the agonist is effective, independently of its pharmacokinetic properties. The invention also provides PTH- and PTHrP-derived polypeptides.

SCREENING METHODS USING G-PROTEIN COUPLED RECEPTORS AND RELATED COMPOSITIONS

Statement as to Federally Funded Research

This invention was made with United States Government support under Grant DK 11794 awarded by the National Institute of Health. The Government has certain rights to this invention.

Background of the Invention

In general, the invention relates to a screening method for agonists of G-protein coupled receptors (GPCRs) with prolonged or short-lived activity. More specifically, the invention is related to parathyroid (PTH) hormone or PTH-related protein (PTHrP) ligand analogs that have either more prolonged or shorter-lived activity on the PTH receptor (PTHR) than does PTH(1-34). The invention also relates PTHR ligands identified using the methods of the invention and uses of such ligands in treating disease.

GPCRs are large group of membrane receptors which, in response to activation by an agonist, activate G-proteins which then, in turn, cause activation of at least one signaling cascade, such as the cyclic AMP/protein kinase A cascade. This large groups of receptors is found in organisms ranging from bacteria to man, and are involved in, for example, hormonal, neuronal, and olfactory signal transduction.

The parathyroid hormone receptor (PTHR) is the endogenous receptor for both PTH and PTH related protein (PTHrP), yet each ligand has a distinct biological function. PTH regulates calcium and phosphate homeostasis and acts as a gland-secreted endocrine hormone on target cells in bone and kidney. PTH also reduces the reabsorption of inorganic phosphate (P_i) largely through its effects on sodium-dependent phosphate transporters (NaP_i-IIa and NaP_i-IIc) located in renal proximal tubule (PT) cells. PTHrP regulates cell proliferation

and differentiation programs in developing tissues, and is secreted and acts in a paracrine fashion within tissue primordia (Kronenberg, H.M. *Ann. N.Y. Acad. Sci.* 1068:1-13 (2006)).

PTH and PTHrP are most homologous in their amino-terminal (residues 1-14) signaling domains (eight amino acid identities), and show moderate homology in their 14-34 binding domains (three identities). It has been generally inferred that the fully active (residues 1-34) portions of PTH and PTHrP interact with the PTHR via largely identical mechanisms (Caulfield et al., *Endocrinology* 127:83-87 (1990); Abou-Samra et al., *Endocrinology* 125:2215-2217 (1989)). This mechanism is thought to consist of two principal components: an interaction between the carboxy-terminal binding domain of the ligand and the amino-terminal extracellular (N) domain of the receptor, and an interaction between the amino-terminal signaling domain of the ligand and the juxtamembrane (J) region of the receptor, which contains the intracellular loops and seven transmembrane helices (Hoare et al., *J. Biol. Chem* 276:7741-7753 (2001); Castro et al., *Proc. Natl. Acad. Sci. USA* 102:16084-16089 (2005); Witelsberger et al., *Biochemistry* 45:2027-2034 (2006); Shimizu et al., *J. Biol. Chem.* 280:1797-1807 (2005); Gensure et al., *Biochem. Biophys. Res. Commun.* 328:666-678 (2005)). However, the extent, if any, to which the precise mechanisms of binding used by the two ligands differ remains to be determined.

In humans, PTH(1-34) has potent, bone-anabolic effects, and induces marked increases in bone mineral density and bone strength. Indeed, recombinant human PTH(1-34) is now considered to be one of the most effective treatments for osteoporosis (Tashjian and Gagel, *J. Bone Miner. Res* 21:354-365 (2006)). Importantly, hPTH(1-34) must be administered in a pulsatile fashion (e.g., once daily subcutaneous injection) in order for its bone-forming effects to be realized. With more prolonged administrations, as with a sustained infusion pump mechanism, PTH(1-34) exerts a net catabolic effect on bone, due to a greater activation of the bone-resorptive responses mediated by the osteoclasts, relative to the bone-forming responses mediated by the

osteoblasts. The duration of exposure of the PTH receptor in bone to a PTH ligand is thus a key determinant of the overall bone-formation response achieved by that ligand, and thus its effectiveness as a treatment for osteoporosis.

Clinical studies have shown that PTHrP(1-36) can also increase bone mineral density in humans, and can do so approximately to the same extent as does PTH(1-34), although higher doses are required (Horwitz et al., *J. Endocrinol. Metab.* 88:569-575 (2003). Importantly, even at such higher doses, PTHrP(1-36) did not stimulate the adverse, bone resorptive and hypercalcemic responses that would be expected for equivalent doses of PTH(1-34) (Horwitz et al., *J. Endocrinol. Metab.* 88:569-575 (2003); Horwitz et al., *J. Bone Miner. Res.* 20:1792-1803 (2005); Horwitz et al., *Osteoporosis Int.* 17:225-230 (2006)). The difference in biological activity of the two peptides is not due merely to a difference in pharamacokinetics. A direct comparison of the two peptides using steady-state infusions methods showed that PTHrP(1-36) is markedly less efficacious than PTH(1-34) for stimulating the renal synthesis of 1,25-$(OH)_2$vitamin D3 (Horwitz et al., *J. Bone. Mineral. Research.* 20:1792-1803 (2005)).

In addition to osteoporosis, hPTH(1-34) has been shown to be effective in treating conditions of PTH deficiency, namely hypoparathyroidism. Thus, PTH(1-34) was shown to be a safe and effective alternative to calcitriol therapy and was able to maintain normal serum calcium levels without hypercalciuria in patients with hypoparathyroidism (Winer et al., *J. Clin. Endocrinol. Metab.* 88:4214-4220 (2003)). The peptide had to be injected at least twice daily, and the authors recognized the need in this disease for a long-acting PTH(1-34) analog (Winer et al., *J. Clin. Endocrinol. Metab.* 88:4214-4220 (2003).

Therefore, there exists a need in the art for PTH or PTHrP analogs that have longer- or shorter-lived actions on the PTH receptor than does PTH(1-34). There also exists a need for assays that allow one to distinguish between PTH peptides that have short- versus long-acting effects.

Summary of the Invention

According to classical GPCR theory, two forms of a G-protein-coupled receptor can be distinguished: a form (RG) that is bound to a G-protein and a form (R) that is not bound to a G-protein. GPCR signaling requires that the G-protein be directly activated by the receptor, i.e., the RG state must form, and this RG formation can be induced by binding of an agonist ligand. Binding of an agonist ligand induces or stabilizes the RG state, and reciprocally, the RG state stabilizes the high affinity binding of an agonist. Upon binding GTP, or, a non-hydrolyzable GTP analog, such as GTPγS, a receptor-coupled G protein will dissociate from the receptor, causing the receptor to revert to a low affinity state. It is now recognized that some GPCRs, like the PTHR, can form a novel state (R^0) that can bind certain agonist ligands with high affinity even in the presence of GTPγS, and hence, even when the receptor is presumably not bound by a G protein. In general, the proportions of a GPCR in a cell that are in the, RG, R, or R^0 state may vary, depending on cell type and conditions. For these reasons, prior work on assessing the binding of ligands to a GPCR generally did not clearly distinguish between the RG, R, or R^0 states. The present inventors, studying the PTH receptor, an exemplary GPCR, have discovered that ligands which bind with high affinity to the R^0 state, in addition to the RG state, have a longer activity half-life than ligands that bind to R^0 with lower affinity, and that this prolonged activity does not depend on the bioavailability or the pharmacokinetics of the ligand in vivo. Correspondingly, agonists with a short duration of action have a lower affinity for the R^0 form of the receptor. Based on this discovery, the invention provides methods for identification of either long-acting or short-acting GPCR agonists, and peptide agonists identified using the methods of the invention.

In a first aspect, the invention provides a method for determining whether a candidate compound is a long-acting agonist of a G protein coupled receptor (GPCR). The method includes (a) contacting the GPCR with the compound, where the GPCR is in the RG form, (b) measuring the affinity of the compound for the RG form of the GPCR, (c) contacting the GPCR with the

compound, where the GPCR is in the R^0 form, and (d) measuring the affinity of the compound for the R^0 form of the GPCR, where a compound that (i) has an affinity for the RG form of the GPCR that is at least 1% (e.g., 5, 10, 25, 30, 50, 60, 75, 90, 100, 125, 150, 200, 150, 300, 400, 500, 750, or 1000%) of an endogenous agonist for the GPCR, and (ii) has a greater affinity (e.g., 1, 5, 10, 25, 50, 100, 200, 500, 1000, 2000, 5000, or 10,000% greater) for the R^0 form of the GPCR than the endogenous agonist or is identified as a long-acting agonist of the GPCR. The method may further include the steps of (e) administering the candidate compound to an animal, and (f) measuring at least one physiological response of the animal to the compound. The receptor may be a human receptor. The GPCR may be a secretin family receptor (e.g., a PTH/PTHrP receptor such as a human PTH/PTHrP receptor). When the receptor is involved in calcium homeostasis or transport, the measuring step (b) or (f) may be performed by measuring intracellular or blood calcium levels. For any GPCR, the affinity-measuring step (b) or step (d) may be performed using a competition binding assay. The competition binding assay may use a ligand that is specific for the RG form or specific for the R^0 form of the GPCR. The measuring step (b) may be performed using a delayed cAMP assay (e.g., as described herein). The R^0 form of the GPCR may enriched using a nonhydrolizable nucleotide analog (e.g., GTPγS). The RG form of the GPCR may be enriched using a dominant-negative G-protein. The receptor may be on a cell or in a membrane. The candidate compound may include a peptide or may be from a chemical library or natural product library.

In another aspect, the invention also features a method for determining whether a candidate compound is a short-acting agonist of a G protein coupled receptor (GPCR). The method includes (a) contacting the GPCR with the compound, where the GPCR is in the RG form, (b) measuring the affinity of the compound for the RG form of the GPCR, (c) contacting the GPCR with the compound, where the GPCR is in the R^0 form; and (d) measuring the affinity of the compound for the R^0 form of the GPCR, where a compound that (i) has an affinity for the RG form of the GPCR that is at least 1% (e.g., 5, 10, 25, 30, 50,

60, 75, 90, 100, 125, 150, 200, 150, 300, 400, 500, 750, or 1000%) of an endogenous agonist for the GPCR, and (ii) has a lower affinity (e.g., 99, 95, 90, 85, 75, 65, 55, 50, 40, 30, 25, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001%) for the R^0 form of the GPCR than the endogenous agonist is identified as a short-acting agonist of the GPCR. The receptor may be a human receptor. The method may further including the steps of (e) administering the candidate compound to an animal, and (f) measuring at least one physiological response of the animal to the compound. The GPCR may be a secretin family receptor (e.g., a PTH/PTHrP receptor such a human PTH/PTHrP receptor). When the receptor is involved in calcium homeostasis or transport, measuring step (b) may be performed by measuring intracellular calcium levels. For any GPCR, the measuring step (b) or step (d) is performed using a competition binding assay (e.g., using a ligand that is specific for the RG form or specific for the R^0 form of the GPCR). The measuring step (b) may be performed using a delayed cAMP assay. In certain embodiments, the R^0 form of the GPCR may be enriched using a nonhydrolizable nucleotide analog (e.g., GTPγS). The RG form of the GPCR may be enriched using a dominant-negative G-protein. The receptor may be on a cell or in a membrane. The candidate compound may include a peptide or may be from a chemical library or a natural product library.

In another aspect the invention features a polypeptide having a low affinity for PTH R^0 (e.g., and a high affinity for RG). The polypeptide may be a short-acting agonist or may be RG selective. The polypeptide may have an amino acid sequence modified by a substitution, deletion and/or addition of one or more (e.g., 2, 3, 4, 5, 6, 7, 8) amino acids relative to the wild-type PTH or PTHrP sequence. The polypeptide may have a histidine at position 5 or an alanine at position 20, 23, 24, or 28. The polypeptide may be Ala^{23}-PTH(1-34), Ala^{23}-PTHrP(1-36), His^5-PTH(1-34), His^5-PTHrP(1-36), or a fragment thereof. The polypeptide may be selected from the group consisting of any of those identified as RG selective in the table of Figure 26B. The polypeptide may be

formulated for pharmaceutical administration (e.g., as described herein) or may be purified.

The invention also features a method for treating osteoporosis in a subject comprising administering the polypeptide of the previous aspect, an RG selective polypeptide (e.g., those described herein), a polypeptide described herein that is a long-acting agonist, or any polypeptide described herein, or a pharmaceutically acceptable form thereof, to the subject in need thereof in an amount sufficient to treat osteoporosis. The invention also features a method for treating fracture repair, osteomalacia, arthritis, thrombocytopenia, hypoparathyroidism or hyperphosphatemia or increasing stem cell mobilization in a subject, comprising administering the polypeptide of the previous aspect or any polypeptide described herein, or a pharmaceutically acceptable form thereof, to the subject in an amount sufficient to treat the disease or condition or to increase stem cell mobilization. The polypeptide or pharmaceutically acceptable form thereof may be administered subcutaneously, intravenously, intranasally, transpulmonarily, transdermally, or orally.

In another aspect, the invention features a polypeptide (PTH analog or PTH derivative) which binds the PTH receptor and has a high affinity for PTH receptor R^0 form. The polypeptide may have an amino acid sequence modified by a substitution, deletion and/or addition of one or more amino acids relative to the wild-type PTH or PTHrP sequence. The polypeptide may also have an arginine at position 19 or an isoleucine at position 5. The polypeptide may be Ala^1,Aib^3[M]PTH(1-28), Ala^1,Aib^3[M]PTH(1-34), or Ile^5-PTHrP(1-36). The polypeptide may be selected from the group consisting of any of the peptides of Figure 26B having an IC_{50} less than or equal to 2.9 nM or 7.9 nM and I^5-hPTHrP(1-36) (#1208), based on the data of Figure 26B. The polypeptide may be formulated for pharmaceutical administration (e.g., as described herein) or may be purified.

The invention also features a method for treating a disease or condition selected from the group consisting of hypoparathyroidism, hyperphosphatemia, tumoral calcinosis, and osteoporosis in a subject, by administering a

polypeptide of the previous aspect, an R^0 selective polypeptide described herein, a polypeptide described herein that is a long-acting agonist, or any polypeptide described herein, or a pharmaceutically acceptable form thereof, to a subject in need thereof in an amount sufficient to treat the disease or condition. The invention also features a method for treating a subject needing fracture repair, or having osteomalacia, arthritis, thrombocytopenia, or requiring stem cell mobilization comprising administering the polypeptide of the previous aspect or any polypeptide described herein, or a pharmaceutically acceptable form thereof, to a subject in an amount sufficient to repair the fracture, to treat the disease, or to mobilize stem cells. The polypeptide or pharmaceutical composition thereof may be administered subcutaneously, intravenously, intranasally, transpulmonarily, transdermally, and orally.

The invention also features a PTH or PTHrP polypeptide having an amino acid sequence modified by a substitution, deletion and/or addition of one or more amino acids relative to the wild-type PTH or PTHrP sequence. The polypeptide may have an arginine at position 19 or an isoleucine at position 5. The polypeptide may be selected from the group consisting of AVAEIQLMHQRGKSIQDLRRRFFLHHLIAEIHTAEI: M-PTH(1-11)/PTHrP(12-36)OH;
AVAEIQLMHQRAKWIQDLRRRFFLHHLIAEIHTAEI: M-PTH(1-14)/PTHrP(15-36)OH;
AVAEIQLMHQRAKWLNSMRRRFFLHHLIAEIHTAEI: M-PTH(1-18)/PTHrP(19-36)OH;
SVSEHQLMHNLGKHIQDLRRRFFLHHLIAEIHTAEI: [H^5]-hPTH(1-14)/PTHrP(15-36)OH;
AVAEIQLMHQRAKWLNSMRRVEWLRKKLQDVHNF: [R^{19}],M-hPTH(1-34)OH; SVSEIQLMHNLGKHIQDLERRFFLHHLIAEIHTAEI: [E^{19}]-hPTH(1-14)/PTHrP(15-36)OH;
AVAEIQLMHQRAKWIQDLERRFFLHHLIAEIHTAEI: [E^{19}],M-hPTH(1-14)/PTHrP(15-36)OH; and
AVAEIQLMHQRAKWLNSMERVEWLRKKLQDVHNF: [E^{19}],M-hPTH(1-

34)OH. The polypeptide may have a histidine at position 5. The polypeptide may be represented by one of the follow formulas Ala^{1},Aib^{3}[M]PTH(1-28), Ala^{23}PTH, and Ile^{5}-PTHrP. The polypeptide may be selected from the group consisting of: AVAEHQLMHQRAKWLNSMERVEWLRKKLQDVHNF: [H^{5},E^{19}],M-PTH(1-34); AVAEHQLMHQRAKWIQDLERRFFLHHLIAEIHTAEI: [H^{5},E^{19}],M-hPTH(1-14)/PTHrP(15-36); SVSEIQLMHNLGKHLNSMERVEFLHHLIAEIHTAEI: hPTH(1-22)/PTHrP(23-36); SVSEIQLMHNLGKHLNSMERVEWLRKKLQDIHTAEI: PTH(1-30)/PTHrP(31-36); AVAEIQLMHQRAKWLNSMERVEALRKKLQDVHNF: [A^{23},E^{19}],M-PTH(1-34); and AVAEIQLMHQRAKWLNSMRRVEALRKKLQDVHNF [A^{23}],M-PTH(1-34). The polypeptide may be used in any treatment methods or any compositions (e.g., pharmaceutical compositions described herein).

In another aspect, the invention features a polypeptide including an amino acid sequence having the formula or including an amino acid sequence substantially identical to an amino acid sequence defined by the formula:

X1-Val-X2-Glu-His-Gln-Lys-Met-His-X3-X4-X5-X6-X7,

wherein:

X1 is Ser, Ala, Gly, or an α-helix stabilizing residue (e.g., Aib);

X2 is Ser, Ala, or an α-helix stabilizing residue (e.g., Aib);

X3 is Asn, Ala, Glu, Val, Asp, or Gln;

X4 is Val, Ala, Trp, Ile, Met, Lys, Arg, Leu, or Har;

X5 is Gly, His, Arg, Ala, or an α-helix stabilizing residue (e.g., Aib);

X6 is Lys, Gln, Leu, His, Trp, Ala, Arg, or an α-helix stabilizing residue (e.g., Aib); and

X7 is Arg, Leu, Phe, Trp, His, or an α-helix stabilizing residue (e.g., Aib);

or a fragment thereof containing amino acids 1-10, 1-11, 1-12, or 1-13, or a pharmaceutically acceptable salt thereof. The α-helix stabilizing residue may be, for example, a non-encoded amino acid such as (2-aminoisobutyric

acid), ACPC (1-aminocyclopropylcarboxylic acid), DEG (diethylglycine), or 1-aminocyclopentanecarboxylic acid. In certain embodiments, the amino acid sequence has 1, 2, 3, 4, 5, 6, 7, or 8 substitutions relative to the corresponding wild-type PTH sequence. In certain embodiments, the polypeptide includes an Ala, Gly, or an α-helix stabilizing residue (e.g., Aib) at X1; an Ala or an α-helix stabilizing residue (e.g., Aib) at X2; an Ala, Glu, Val, Asp, or Gln at X3; a Val, Ala, Trp, Ile, Met, Lys, Arg, or Har at X4; a His, Arg, Ala, or an α-helix stabilizing residue (e.g., Aib) at X5; a Gln, Leu, His, Trp, Ala, Arg, or an α-helix stabilizing residue (e.g., Aib) at X6; an Arg, Leu, Phe, Trp, or an α-helix stabilizing residue (e.g., Aib) at X7; or a combination thereof. In any of these embodiments, the polypeptide may have an amino acid sequence fewer than 100, 50, 36, 34, 30, 25, or 20 in length (e.g., 10-14 amino acids). In certain embodiments, the polypeptide is 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, or 10 amino acids in length. The polypeptide may be part of a composition including a pharmaceutically acceptable carrier.

In another aspect, the invention features a polypeptide including an amino acid sequence of the formula, or includes an amino acid sequence substantially identical to an amino acid sequence defined by the formula:

X1-Val-X2-Glu-X3-Gln-Leu-Met-His-X4-X5-X6-X7-X8-Leu-Asn-Ser-Met-Glu-X9-Val-Glu-X10-X11-Arg-Lys-Lys-X12,

wherein:

X1 is Ser, Ala, or an α-helix stabilizing residue (e.g., Aib);

X2 is Ser, Ala, or an α-helix stabilizing residue (e.g., Aib);

X3 is Ile or His;

X4 is Asn, Glu, Val, Asp, or Gln;

X5 is Val, Ala, Trp, Ile, Met, Lys, Arg, Leu, or Har;

X6 is Gly, His, Arg, or Ala;

X7 is Lys, Gln, Leu, His, Trp, Ala or Arg;

X8 is Arg, Leu, Phe, Trp, His, or Ser;

X9 is Arg or Ala;

X10 is Trp, Ala or Phe;

X11 is Leu or Ala; and

X12 is Leu or Ala;

and wherein the amino acid sequence comprises at least one of the amino acids selected from the group consisting of His at position X3, Ala at position X9, Ala at position X10, Ala at position X11, and Ala at position X12, a fragment thereof comprising amino acids 1-24, 1-25, 1-26, or 1-27 of said amino acid sequence, or a pharmaceutically salt thereof. The polypeptide may bind with low affinity to the R^0 form of a PTH receptor (e.g., bind with high affinity to the RG form of the PTH receptor). The polypeptide may be RG selective or may be a short-acting agonist of the receptor. The polypeptide may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions relative to the corresponding wild type sequence. In certain embodiments, the polypeptide includes an Ala or an α-helix stabilizing residue (e.g., Aib) at X1; an Ala or an α-helix stabilizing residue (e.g., Aib) at X2; an His at X3; a Glu, Val, Asp, or Gln at X4; a Val, Ala, Trp, Ile, Met, Lys, Arg, or Har at X5; a His, Arg, or Ala at X6; a Gln, Leu, His, Trp, Ala, or Arg at X7; an Arg, Leu, Phe, Trp, or Ser at X8; an Ala at X9; an Ala or Phe at X10; an Ala at X11; an Ala at X12; or a combination thereof. The polypeptide may be fewer than 100, 75, 60, 50, 40, 36, 34, 33, 32, 31, 30, 29, or 28 amino acids in length. The polypeptide may be 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acids in length (e.g., 24-28 amino acids in length). In certain embodiments at least one (e.g., 2, 3, or 4) of X9, X10, X11, or X12 is alanine.

In another aspect, the invention features a polypeptide including an amino acid sequence of the formula, or substantially identical to an amino acid sequence defined by the formula:

X1-Val-X2-Glu-Ile-Gln-Leu-Met-His-X3-X4-X5-X6-X7-Leu-Asn-Ser-Met-Arg-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu,

wherein

X1 is Ser, Ala, or Aib;

X2 is Ser, Ala, or Aib;

X3 is Asn, Glu, Val, Asp, or Gln;

X4 is Val, Ala, Trp, Ile, Met, Lys, Arg, or Leu;

X5 is Gly, His, Arg, or Ala;

X6 is Lys, Gln, Leu, His, Trp, Ala, or Arg; and

X7 is Arg, Leu, Phe, Trp, His, or Ser,

or a fragment thereof containing amino acids 1-24, 1-25, 1-26, or 1-27 of said amino acid sequence, or a pharmaceutically acceptable salt thereof. The polypeptide may be R^0 selective or may be a long-acting PTH agonist. The amino acid sequence may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions (e.g., at any of the positions described above relative to the wild type PTH sequence). In certain embodiments, the polypeptide includes an Ala or Aib at X1; an Ala or Aib at X2; a Glu, Val, Asp, or Gln at X3; a Val, Ala, Trp, Ile, Met, Lys, or Arg at X4; a His, Arg, or Ala at X5; a Gln, Leu, His, Trp, Ala, or Arg at X6; an Arg, Leu, Phe, Trp, or Ser at X7; or a combination thereof. The polypeptide may be fewer than 100, 75, 60, 50, 40, 36, 34, 33, 32, 31, 30, 29, or 28 amino acids in length. The polypeptide may be 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acids in length (e.g., 24-28 amino acids in length). The polypeptide may be in a composition with a pharmaceutically acceptable carrier.

In another aspect, the invention features a polypeptide comprising an amino acid sequence having the formula, or an amino acid sequence substantially identical to a polypeptide defined by the formula:
Ala-Val-Ser-Glu-His-Glu-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-X1-Arg-Arg-Arg-X2-Phe-Leu-X3-X4-Leu-Ile-X5-X6-X7-X8-X9-X10-Glu-Ile

wherein:

X1 is Leu, Ala, Ser, Met, Phe, or Glu;

X2 is Phe, Ala, Ser, Leu, Asn, Trp, Glu, or Lys;

X3 is His, Leu, Arg, Lys, Trp, Ile, or Phe;

X4 is His, Ala, Ser, Asn, Lys, or Arg;

X5 is Ala, Gly, Ser, Asn, Gln, Trp, Glu, or Lys;

X6 is Glu, Gly, Ser, Leu, Asn, Asp, Lys, or Ala;

X7 is Ile, Leu, Val, Lys, or Ala;

X8 is His or Ala

X9 is Thr, Asn, or Ala; and

X10 is Ala or Phe,

or a fragment thereof containing amino acids 1-24, 1-25, 1-26, 1-27, 1-28, 1-29, 1-30, 1-31, 1-32, 1-33, 1-34, or 1-35 of said amino acid sequence, and wherein said polypeptide comprises at least one amino acid substitution as compared to the corresponding wild type PTHrP sequence or a fragment thereof; or a pharmaceutically acceptable salt thereof. The polypeptide may bind with low affinity to the R^0 form of a PTH receptor (e.g., bind with high affinity to the RG form of the PTH receptor). The polypeptide may be RG selective or may be a short-acting agonist of the PTH receptor. The polypeptide may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions relative to the corresponding wild type PTHrP sequence. In certain embodiments, the polypeptide has an Ala, Ser, Met, Phe, or Glu at X1; an Ala, Ser, Leu, Asn, Trp, Glu, or Lys at X2; a Leu, Arg, Lys, Trp, Ile, or Phe at X3; an Ala, Ser, Asn, Lys, or Arg at X4; Gly, Ser, Asn, Gln, Trp, Glu, or Lys at X5; a Gly, Ser, Leu, Asn, Asp, Lys, or Ala X6; a Leu, Val, Lys, or Ala at X7; an Ala at X8; an Asn or Ala at X9; a Phe at X10; or a combination thereof. In particular embodiments, the polypeptide has an Ala or Glu at X1, an Ala at X2, a Leu at X3, a Lys at X4, or a combination thereof. The polypeptide may be fewer than 100, 75, 60, 50, 40, 36, 34, 33, 32, 31, 30, 29, or 28 amino acids in length. The polypeptide may be 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acids in length (e.g., 28-36 amino acids in length). The polypeptide may have a free hydroxyl or be amidated at its C-terminus. The polypeptide may include a sequence selected from the amino acid sequences of Table 1, or be substantially identical to such sequences. The polypeptide may be in a composition with a pharmaceutically acceptable carrier.

Table 1

A18-PTHrP(1-28)	A18,26,W22-PTHrP(1-28)	A18,22,K26-PTHrP(1-31)
S18-PTHrP(1-28)	A18,W22,K26-PTHrP(1-28)	E18,A22,K27-PTHrP(1-31)
M18-PTHrP(1-28)	E18,A22,K26-PTHrP(1-28)	A18,22,L25,K26-PTHrP(1-31)
F18-PTHrP(1-28)	E18,S22,A26-PTHrP(1-28)	E18,A22,L25,K26-PTHrP(1-31)
E18-PTHrP(1-28)	E18,N22,A26-PTHrP(1-28)	E18,A22,L25,K26-PTHrP(1-31)
A22-PTHrP(1-28)	E18,N22,K26-PTHrP(1-28)	E18,A22,L25,K26,G29-PTHrP(1-31)
S22-PTHrP(1-28)	E18,L22,A26-PTHrP(1-28)	E18,A22,L25,K26,S29-PTHrP(1-31)
L22-PTHrP(1-28)	E18,L22,K26-PTHrP(1-28)	E18,A22,L25,K26,N29-PTHrP(1-31)
N22-PTHrP(1-28)	E18,W22,A26-PTHrP(1-28)	E18,A22,L25,K26,Q29-PTHrP(1-31)
W22-PTHrP(1-28)	E18,W22,K26-PTHrP(1-28)	E18,A22,L25,K26,W29-PTHrP(1-31)
E22-PTHrP(1-28)	E18,K22,A26-PTHrP(1-28)	E18,A22,L25,K26,E29-PTHrP(1-31)
K22-PTHrP(1-28)	E18,K22,26-PTHrP(1-28)	E18,A22,L25,K26,K29-PTHrP(1-31)
A26-PTHrP(1-28)	E18,A22,26-PTHrP(1-28)	E18,A22,L25,K26,G30-PTHrP(1-31)
S26-PTHrP(1-28)	A18,22,L25,K26-PTHrP(1-28)	E18,A22,L25,K26,S30-PTHrP(1-31)
N26-PTHrP(1-28)	A18,22,K25,26-PTHrP(1-28)	E18,A22,L25,K26,L30-PTHrP(1-31)
K26-PTHrP(1-28)	A18,22,I25,K26-PTHrP(1-28)	E18,A22,L25,K26,N30-PTHrP(1-31)
R26-PTHrP(1-28)	A18,22,W25,K26-PTHrP(1-28)	E18,A22,L25,K26,D30-PTHrP(1-31)
L25-PTHrP(1-28)	A18,22,F25,K26-PTHrP(1-28)	E18,A22,L25,K26,K30-PTHrP(1-31)
W25-PTHrP(1-28)	A18,S22,L25,K26-PTHrP(1-28)	E18,A22,L25,K26,S31-PTHrP(1-31)
K25-PTHrP(1-28)	A18,S22,K25,26-PTHrP(1-28)	E18,A22,L25,K26,L31-PTHrP(1-31)
R25-PTHrP(1-28)	E18,A22,L25,K26-PTHrP(1-28)	E18,A22,L25,K26,V31-PTHrP(1-31)
A18,22,26-PTHrP(1-28)	E18,A22,K25,26-PTHrP(1-28)	E18,A22,L25,K26,K31-PTHrP(1-31)
A18,22,K26-PTHrP(1-28)	E18,S22,L25,K26-PTHrP(1-28)	E18,A22,L25,K26-PTHrP(1-34)
A18,26,S22-PTHrP(1-28)	E18,S22,K25,26-PTHrP(1-28)	E18,A22,L25,K26,A30-PTHrP(1-34)
A18,S22,K26-PTHrP(1-28)	A18,22,K26-PTHrP(1-30)	E18,A22,L25,K26,A31-PTHrP(1-34)
A18,26,N22-PTHrP(1-28)	E18,A22,K27-PTHrP(1-30)	E18,A22,L25,K26,A32-PTHrP(1-34)
A18,N22,K26-PTHrP(1-28)	A18,22,L25,K26-PTHrP(1-30)	E18,A22,L25,K26,A33-PTHrP(1-34)
A18,26,L22-PTHrP(1-28)	E18,A22,L25,K26-PTHrP(1-30)	E18,A22,L25,K26,Q29,D30,V31,N33
A18,L22,K26-PTHrP(1-28)		,F34-PTHrP(1-34)

In another aspect, the invention features a PTH or PTHrP polypeptide (e.g., of any of the above aspects or described herein) where the N-terminus is substituted with a bulky residue (e.g., Trp). Such polypeptides include Trp^1-PTH(1-34), Trp^1-M-PTH(1-34), and TRP^1-PTHrP(1-36), or a fragment thereof containing amino acids 1-10, 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26, 1-27, 1-28, 1-29, 1-30, 1-31, 1-32, 1-33, 1-34, or 1-35 of said sequence. The polypeptide may have reduced (e.g., by at least 1, 5, 10, 25, 50, 75, 90, 95, 99, 99.5, 99.9, 99.95, or 99.99%) PLC signaling activity at the PTH receptor as compared to the polypeptide lacking the bulky residue substitution. Other bulky residues include Phe, Tyr, and *p*-benzoylphenylalanine (Bpa). In certain embodiments, the polypeptide includes any one (e.g., 2, 3, 4, 5, 6, or 7) of the mutations set forth in the M or Mc modifications, where M represents

[Ala^{1,12},Aib^{3},Gln^{10},homoarginine^{11},Trp^{14},Arg^{19}] and Mc represents Ala^{1,3,12},Gln^{10},Arg^{11},Trp^{14}, Arg^{19} PTH sequence, or any combination thereof. Hybrid peptides may further include a substitution at position 5 (e.g., a histidine at position 5). Exemplary polypeptides include Trp^{1}-PTH(1-28) and Trp^{1}-M-PTH(1-28).

In another aspect of the invention, the invention features a polypeptide including a hybrid PTH/PTHrP polypeptide or a polypeptide including an amino acid sequence substantially identical to a hypbrid PTH/PTHrP polypeptide. The polypeptide may be represented by the formula PTH(1-X)/PTHrP(Y-36), where X is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, or 34 and Y = X+1. In certain embodiments, the hybrid polypeptide contains any one (e.g., 2, 3, 4, 5, 6, or 7) of the mutations set forth in the M or Mc modifications, where M represents [Ala^{1,12},Aib^{3},Gln^{10},homoarginine^{11},Trp^{14},Arg^{19}] and Mc represents Ala^{1,3,12},Gln^{10},Arg^{11},Trp^{14}, Arg^{19} PTH sequence, or any combination thereof. Hybrid peptides may further include a substitution at position 5 (e.g., a histidine at position 5).

In any of the polypeptides described above, the polypeptide may be biologically active, e.g., have an affinity for the RG form of the GPCR that is at least 1% (e.g., 5, 10, 25, 30, 50, 60, 75, 90, 100, 125, 150, 200, 150, 300, 400, 500, 750, or 1000%) of an endogenous agonist for the GPCR, and have a lower affinity (e.g., 99, 95, 90, 85, 75, 65, 55, 50, 40, 30, 25, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001%) for the R^0 form as compared to a control (e.g., an endogenous ligand for the GPCR). In other embodiments, the polypeptide has an affinity for the RG form of the GPCR that is at least 1% (e.g., 5, 10, 25, 30, 50, 60, 75, 90, 100, 125, 150, 200, 150, 300, 400, 500, 750, or 1000%) of an endogenous agonist for the GPCR, and (ii) has a greater affinity (e.g., 1, 5, 10, 25, 50, 100, 200, 500, 1000, 2000, 5000, or 10,000% greater) for the R^0 form of the GPCR than the endogenous agonist or is identified as a long-acting agonist of the GPCR. In the above aspects, the polypeptide may be RG selective, R^0 selective, a short-acting agonist, or a

long-acting agonist. In certain embodiments, the polypeptide may be modified (e.g., acetylated at the N-terminal, amidated at the C-terminal, or contain any of the modifications described herein).

The invention also features a nucleic acid including a sequence encoding a polypeptide described herein (e.g., those described above). The nucleic acid may be operably linked to promoter and/or part of a vector. The invention also features a cell (e.g., a prokaryotic cell such as bacterial cell or a eukaryotic cell such as yeast or mammalian, for example, human, cell) including the vector. The invention also features a method of making the polypeptide by growing the cell under conditions which induce expression of said nucleic acid and optionally purifying said polypeptide.

By "GPCR" is meant any polypeptide comprising a G protein coupled receptor or functional fragment thereof. Desirably, a GPCR has at least 70%, 80%, 90%, 95%, 99%, or 100% sequence identity to a naturally occurring GPCR. Exemplary GPCRs are described herein.

By "RG form" of a GPCR is meant the G-protein-bound receptor conformation. The RG form of a GPCR can be induced, for example, by increased G-protein binding of the GPCR. In the assays of the invention, at least 1%, 5%, 10%, 25%, 50%, 75%, 90%, 95%, or 99% of the receptors are in the RG form when affinity for RG form is measured.

By "R^0 form" of a GPCR is meant the receptor conformation that occurs when the GPCR is not bound to a G-protein, but is capable of binding at least some ligands of the receptor. The R^0 form of a GPCR, relative to RG, can be favored, for example, by preventing or reducing G-protein binding to the GPCR. In the assays of the invention, at least 0.1%, 1%, 5%, 10%, 25%, 50%, 75%, 90%, 95%, or 99% of the receptors may be in the R^0 form when affinity for the R^0 form is measured.

By "affinity" is meant the ability of a compound to interact with a target receptor. In the assays and polypeptides of the invention, affinity may be measured directly by binding (e.g., competition binding assays or FRET), or indirectly through an activity assay (e.g., cAMP signaling or changes in

intracellular calcium). Desirably the compound has an affinity for the receptor of at least 10 μmol, 1 μmol, 500 nmol, 100 nmol, 50 nmol, 25 nmol, 10 nmol, 5 nmol, 1 nmol, 500 pmol, 200 pmol, 100 pmol, 50 pmol, 25 pmol 10 pmol, or 1 pmol as measured by EC_{50} for the RG form or the R^0 form of the GPCR.

By "long-acting agonist" is meant an agonist whose activity (e.g., measured in vivo or in vitro) has a half life that is at least 5%, 10%, 25%, 50%, 75%, 100%, 150%, 200%, 500%, 1000%, or 5000% longer as compared to an endogenous agonist for the same receptor.

By "short-acting agonist" is meant an agonist whose activity (e.g., measured in vivo or in vitro using an assay described herein) has a half life that is less than 95%, 90%, 75%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or 1% as compared to an endogenous agonist for the same receptor.

By "RG selective agonist" is an agonist that exhibits increased binding to the RG form of a receptor relative to the R^0 form of the receptor, as compared to a control agonist (e.g., an endogenous agonist). Receptor selectivity can be expressed as a ratio of binding constants between each receptor form, e.g., R^0/RG ratio, where an increase in this ratio indicates stronger binding to the RG form. As shown in Figures 26A and 26B, the R^0/RG ratio of PTH(1-34) is 67 and the relatively more RG selective PTHrP(1-36) is 260 in binding the human PTH receptor expressed on COS-7 cell membranes. An RG selective agonist may have an R^0/RG ratio of at least 100, 150, 200, 250, 300, 400, 500, 1000, 2000, 3000, 5000, 7000, 10,000, 15,000, 20,000, or 50,000 in this system. The R^0/RG ratio may be at least 1.5, 2, 3, 4, 5, 10, 15, 25, 50, 75, or 100-fold that of the control agonist.

By "R^0 selective agonist" is an agonist that exhibits decreased binding to the RG form of a receptor relative to the R^0 form of the receptor, as compared to a control agonist (e.g., an endogenous agonist). Receptor selectivity can be expressed as a ratio of binding constants between each receptor form, e.g., R^0/RG ratio, where a dcrease in this ratio indicates stronger binding to the R^0 form. As shown in Figures 26A and 26B, the R^0/RG ratio of PTH(1-34) is 67 and the relatively more RG selective PTHrP(1-36) is 260 in binding the human

PTH receptor expressed on COS-7 cell membranes. The R^0 selective agonist may have an R^0/RG ratio of less than 60, 50, 40, 30, 25, 20, 25, 10, 5, 2, 1, 0 in this system. The R^0/RG ratio thus may be less than 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.08, 0.05, 0.03, 0.01, 0.008, 0.005, 0.003, or 0.001-fold of that the control agonist.

By "endogenous agonist" of a GPCR is meant a compound produced by an organism, or a synthetic phenocopy of that compound, i.e., a compound having the same pharmacological activity as the endogenous agonist. For example, the native PTH peptide is 1-84, and PTHrP is ~1-140 amino acids; phenocopies of these ligands include PTH(1-34) and PTHrP(1-36), respectively. An endogenous agonist is involved in or modulates the normal physiological activation of the GPCR. Some GPCRs have multiple endogenous agonists (e.g., endogenous agonists for the PTHR include PTH and PTHrP); for purposes of the invention, any endogenous agonist may be used to determine whether the candidate compound is short-acting or long-acting.

By "peptide" or "polypeptide" is meant a chain of amino acids of at least 4, 6, 10, 25, 50, 100, 150, 200, 500, or 1000 amino acids.

By "fragment" of a polypeptide is meant a portion of a sequence at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 amino acids in length

By "subject" is meant either a human or non-human animal (e.g., a mammal).

By "an amount sufficient to treat" is meant an amount sufficient to reduce, prevent, or eliminate at least one symptom associated with the disease or condition.

By a "purified polypeptide" or "isolated polypeptide" is meant a polypeptide that has been separated from other components. Typically, the polypeptide is substantially pure when it is at least 30%, by weight, free from other components. In certain embodiments, the preparation is at least 50%, 60%, 75%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% by weight, free from other components. A purified polypeptide may be obtained, for example, by

extraction from a natural source; by expression of a recombinant polynucleotide encoding such a polypeptide; or by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "biologically active" is meant that the compound or composition (e.g., a polypeptide described herein) has at least one biologically significant effect upon administration to a cell or animal (e.g., a human or non-human mammal). Biological activities of PTH, PTHrP, and analogs thereof (e.g., those described herein) include receptor binding, cAMP or IP_3 production, protein kinase A, protein kinase C, phospholipase C, phospholipase D, and phospholipase A_2 activation, changes (e.g., increases or decreases) in intracellular, plasma, or urinary calcium or phosphate levels, and changes in bone metabolism or catabolism in vivo or in vitro. A biologically active peptide of the invention (e.g., any peptide described herein), for example, may exhibit increases (e.g., at least 5%, 10%, 25%, 50%, 100%, 500%, 1000%, 10,000%) or decreases (e.g., 95%, 90%, 75%, 50%, 25%, 10%, 5%, 1%, 0.1%, 0.01%, or 0.001%) in any biological activity as compared to an appropriate control (e.g., a wild-type peptide or a phenocopy thereof such as PTH(1-34) or PTHrP(1-36)).

By "substantially identical" is meant a nucleic acid or amino acid sequence that, when optimally aligned, for example, using the methods described below, share at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with a second nucleic acid or amino acid sequence, e.g., an PTH or PTHrP sequence or fragment thereof. "Substantial identity" may be used to refer to various types and lengths of sequence, such as full-length sequence, epitopes or immunogenic peptides, functional domains, coding and/or regulatory sequences, exons, introns, promoters, and genomic sequences. Percent identity between two polypeptides or nucleic acid sequences is determined in various ways that are within the skill in the art, for instance, using publicly available computer software such as

Smith Waterman Alignment (Smith et al., *J. Mol. Biol.* 147:195-7 (1981)); "Best Fit" (Smith and Waterman, Advances in Applied Mathematics, 482-489 (1981)) as incorporated into GeneMatcher Plus™, Schwarz and Dayhof (1979) Atlas of Protein Sequence and Structure, Dayhof, M. O., Ed pp 353-358; BLAST program (Basic Local Alignment Search Tool; (Altschul et al., *J. Mol. Biol.* 215: 403-10 (1990)), BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, or Megalign (DNASTAR) software. In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared. In general, for proteins, the length of comparison sequences will be at least 6 or 8 amino acids, preferably 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, or 500 amino acids or more up to the entire length of the protein. For nucleic acids, the length of comparison sequences will generally be at least 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 93, 96, 99, 102, 105, 108, 111, 125, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1100, 1200, or at least 1500 nucleotides or more up to the entire length of the nucleic acid molecule. It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "bulky amino acid" is meant any amino acid with a molecular weight greater than 100 Da (e.g., greater than 125, 150, 175, 200, 225, 250, 300, or 400). The molecular weight of each coding amino acid is as follows. Ala: 71.09, Arg: 156.19, Asp: 115.09, Asn: 114.11, Cys: 103.15, Glu: 129.12, Gln: 128.14, Gly: 57.05, His: 137.14, Ile: 113.16, Leu: 113.16, Lys: 128.17, Met:

131.19, Phe: 147.18, Pro: 97.12, Ser: 87.08, Thr: 101.11, Trp: 186.12, Tyr: 163.18, and Val: 99.14.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

Figures 1A-1C are graphs showing dissociation of PTH and PTHrP analogs from the human PTH receptor (PTHR) and the effects of GTPγS. The radioligands ^{125}I-[Nle^{8,21},Tyr^{34}]rPTH(1-34)NH$_2$ (Figure 1A), ^{125}I-[Tyr^{36}]PTHrP(1-36)NH$_2$ (Figure 1B) and ^{125}I-[Ile^{5},Tyr^{36}]PTHrP(1-36)NH$_2$ (Figure 1C) were pre-bound to the human PTHR in membranes prepared from HKRK-B7 cells for 90 minutes; then dissociation was initiated (t=0) by the addition of an excess of the unlabeled analog (5 x 10^{-7} M), added either alone (filled circles) or together with GTPγS (5 x 10^{-5} M, open circles). At each time point, aliquots were removed from the reaction tubes and immediately subjected to rapid vacuum filtration using a 96-well vacuum filtration plate to separate bound from free radioactivity. Non-specific binding was determined in tubes containing the unlabeled ligand (5 x 10^{-7} M) during both the pre-incubation and dissociation phases. The specifically bound radioactivity (SB) at each time point was then expressed as the percent of the specific binding observed at t = 0. Aggregate data from four (Figure 1A), five (Figure 1B), or three (Figure 1C) experiments are shown. Curves were fit to the data using either a two-phase (Figures 1A and 1B) or single phase (Figure 1C) exponential decay equation.

Figures 2A and **2B** are graphs showing GTPγS sensitivity of PTH and PTHrP analog binding to the human and rat PTHRs. Radioligand analog binding to the PTHR in membranes prepared from HKRK-B7 (Figure 2A) or ROS 17/2.8 cells (Figure 2B) was assessed under near-equilibrium conditions in the absence or presence of varying concentrations of GTPγS. Data are expressed as a percent of radioactivity specifically bound (SB) in the absence

of GTPγS. Data in Figure 2A are means (±s.e.m.) from three (PTH(1-34)) or five (PTHrP(1-36) analogs) experiments, and those in Figure 2B are from six experiments, each performed in duplicate. The radioligands studied were ^{125}I-[Nle^{8,21},Tyr^{34}]PTH(1-34)NH_2; [Tyr^{36}]PTHrP(1-36)NH_2; [Ile^5,Tyr^{36}]PTHrP(1-36)NH_2 and [Aib^{1,3},Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]hPTH(1-15)NH_2.

Figures 3A-3D are graphs showing binding of PTH and PTHrP analogs to the G protein-coupled and G protein-uncoupled conformations of the hPTHR. The binding of unlabeled PTH and PTHrP analogs to the G protein-coupled PTHR conformation (RG) and G protein-uncoupled PTHR conformation (R^0) was assessed by competition methods using membranes prepared from transiently transfected COS-7 cells. To assess binding to RG, the cells were co-transfected with the hPTHR and a negative-dominant Gαs subunit ($G\alpha^{ND}$); and ^{125}I-[Aib^{1,3},M]PTH(1-15)NH_2 was used as a tracer radioligand. To assess binding to R^0, the cells were transfected with the hPTHR alone, ^{125}I-[Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2 was used as a tracer radioligand, and the binding reactions were performed in the presence of GTPγS. The unlabeled ligands used were [Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2 (Figure 3A); [Tyr^{36}]hPTHrP(1-36)NH_2 (Figure 3B); [His^5,Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2 (Figure 3C); and [Ile^5,Tyr^{36}]hPTHrP(1-36)NH_2 (Figure 3D). Whereas each ligand binds with relatively high affinity to RG, PTHrP(1-36), and His^5-PTH(1-34) bind with considerably lower affinity to R^0 than do PTH(1-34) and Ile^5-PTHrP(1-36), and thus exhibit stronger RG selectivity. Data are means (±s.e.m.) of three to seven experiments, each performed in duplicate (see also Table 5).

Figures 4A-4D are graphs showing fluorescent resonance energy transfer (FRET) analysis of ligand binding to the PTHR in HEK-293 cells. HEK-293 cells stably transfected with a PTHR construct (PTHR-cam) containing cyan fluorescent protein (CFP) in the third intracellular loop and yellow fluorescent protein (YFP) in the carboxy-terminal tail, were used to assess the kinetics of ligand binding to, and dissociation from the PTHR. With PTHR-cam, excitation of the CFP with ultraviolet light (λ_{exc} = 436 nm)

produces an intramolecular FRET to the YFP, which is observable as an increase in light emission from YFP (λ_{emm} = 535 nm) and a decrease in light emission from CFP λ_{emm} = 480 nm). This FRET signal occurs in the ground-state receptor and decreases upon agonist binding. In each panel, the trace shows the ratio of the fluorescence signals ($F_{YFP(535)}/F_{CFP(480)}$, normalized for channel spill-over) obtained over time in cells superfused with buffer alone or with buffer containing a PTH peptide ligand (times of peptide addition indicated by the black bars above each trace). The ligands used were hPTH(1-34) (Figure 4A); [Aib^{1,3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]rPTH(1-14)NH_2 (Figure 4B); [Tyr^{36}]hPTHrP(1-36)NH_2(Figure 4C), and [Ile^5,Tyr^{36}]hPTHrP(I-36)NH_2 (Figure 4D). The onset of the FRET signal induced by PTHrP(1-36) was slower than that induced by the three other analogs. The signals induced by PTH(1-14) and PTHrP(1-36) analogs decayed upon ligand removal, whereas those induced by PTH(1-34) and Ile^5-PTHrP(1-36) analogs remained stable. Data are from a single experiment, and identical results were obtained in at least three others.

Figures 5A and **5B** are graphs showing the duration of cAMP-signaling responses induced by PTH and PTHrP analogs in cells stably expressing the human PTHR. The duration of cAMP responses induced by PTHrP(1-36) or Ile^5-PTHrP(1-36) in HKRK-B7 cells (950,000 hPTHRs/per cell) was assessed by time course experiments (Figure 5A). The cells were pre-treated for 10 minutes with either buffer alone (basal) or buffer containing ligand (100 nM); at t = 0, the cells were washed, incubated in buffer for the times indicated (wash-out phase), treated with 3-isobutyl-1-methylxanthine (IBMX) for five minutes, and then assessed for intracellular cAMP. The maximum response to each peptide, assessed by incubating cells concomitantly with peptide and IBMX and omitting the wash-out phase, was 185116 and 198118 pmoles/well for PTHrP(1-36) and Ile^5-PTHrP(1-36), respectively. The cAMP level in cells treated with IBMX in the absence of ligand was 2.0±0.3 pmole/well. Data are means (±s.e.m.) of three experiments, each performed in duplicate. In these experiments, PTH(1-34) was also analyzed and induced responses at each time

point that were not different from those induced by PTHrP(1-36). Analogs were similarly assessed in HKRK-B64 cells (90,000 hPTHRs/cell) at a single time-point, 60 minutes after ligand wash-out (Figure 5B). For each peptide, the data are expressed as a percentile of the maximum cAMP responses (indicated in side panel) produced in cells treated concomitantly with that ligand and IBMX for 10 minutes and omitting the wash-out phase. Data are means (±s.e.m) of four experiments, each performed in triplicate. Asterisks indicate statistical analyses of paired responses: PTHrP(1-36) vs. Ile^5-PTHrP(1-36) (Figure 5A), or as indicated by brackets (Figure 5B): *, $P \leq 0.05$; **, $P \leq 0.003$.

Figures 6A-6D are graphs showing binding of PTH and PTHrP analogs to the G protein-coupled and G protein-uncoupled conformations of the hPTHR. Binding reactions were performed as described above for Figures 3A-3D. The unlabeled ligands used were hPTH(1-34)NH_2 (Figure 6A); [$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]rPTH(1-15)NH_2 (Figure 6B); [His^5]hPTH(1-34)NH_2 (Figure 6C); hPTHrP(1-36)NH_2 (Figure 6D). Data are means (±s.e.m.) of three or five experiments, each performed in duplicate (Table 6).

Figures 7A and **7B** shows a dose-response analysis of analog signaling potency. The capacity of PTH and PTHrP ligands to stimulate cAMP formation was assessed in HKRK-B64 cells (Figure 7A). Cells were treated for 30 minutes at room temperature with varying concentrations of ligand in the presence of IBMX. The capacity of the ligands to stimulate the production of inositol phosphates (IPs) was assessed in COS-7 cells transiently transfected with the hPTHR (Figure 7B). Cells were treated for 30 minutes at room temperature with varying concentrations of ligand. The ligands used were [$Nle^{8,21}$,Tyr^{34}]rPTH(1-34)NH_2; [His^5,$Nle^{8,21}$,Tyr^{34}]rPTH(1-34)NH_2; [Tyr^{36}]hPTHrP(1-36)NH_2 and [Ile^5,Tyr^{36}]hPTHrP(1-36)NH_2. Data are means (± s.e.m.) of four (Figure 7A) or five (Figure 7B) experiments, each performed in duplicate. The EC_{50} and Emax values are reported in Table 6 and were not significantly different between peptides, with the exception of the cAMP EC_{50} values for H^5-PTH(1-34) and PTH(1-34) analogs ($P = 0.02$).

Figure 8 is a graph showing cAMP dose responses in rat cells. Rat osteoblastic cells treated with hPTH(1-28)NH_2; $Ala^{1,12}$,Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2; hPTH(1-34)NH_2, or r(rat)PTH(1-34)NH_2. The resulting intracellular cAMP formed was quantified by radioimmuno assay. EC50 values are listed below the graph. Curve fits were obtained by non-linear regression analysis.

Figures 9A-9D are graphs showing in vivo plasma cAMP levels in mice treated with PTH analogs. Wild-type mice were injected subcutaneously with vehicle (0.9% NaCl/0.05% Tween-20), or vehicle containing a PTH peptide at a dose-level of 10 to 1,000 nmol of peptide per kg of body weight, and at indicated times after injection, blood was withdrawn from the tail vein, and the amount of cAMP in the resulting plasma was quantified by radioimmuno assay. Each curve corresponds to a peptide at a defined concentration, as indicated in the graph keys. The plasma cAMP concentrations are plotted as picomole per µl plasma. The data show that at 50 nmol/kg, $Ala^{1,12}$,Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2 (Aib-50, Figure 9A) and hPTH(1-34)NH_2 ((1-34)-50, Figure 9B) produce comparable increases in plasma cAMP concentrations, whereas 1,000 nmol/kg of hPTH(1-28)NH_2 is required to achieve the same increase ((1-28)-1000, Figure 9C, also Figure 9D).

Figures 10A and **10B** are graphs showing in vivo plasma phosphate and serum ionized calcium levels in mice treated with PTH analogs. Wild-type mice were injected subcutaneously with vehicle (0.9% NaCl/0.05% Tween-20), or vehicle containing $Ala^{1,12}$,Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2 or hPTH(1-34)NH_2 at a dose level of 50 nanomoles per kg of body weight, or hPTH(1-28)NH_2 at a dose level of 1,000 nanomoles per kg of body weight and at the indicated times concentrations of plasma phosphate (Figure 10A) and serum ionized calcium (Figure 10B) were determined. Serum ionized calcium concentrations were determined using a Chiron Diagnostics Model 634 Ca^{++}/pH analyzer. Data in A are means (± s.e.m.) of one experiment using six mice (n = 6) for each injection condition; similar results were obtained in three

other experiments. Data in B are means (± s.e.m.) of two experiments, each performed using triplicate mice (n = 3) for each injection condition.

Figure 11 is a graph showing the time courses of phosphate uptake inhibition in opossum kidney cells for PTH(1-34), PTHrP(1-36) and the long-acting PTH(1-28) analog, $Ala^{1,12}$,Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2. Data at each time point are plotted as a percentile of the amount of ^{32}P radioactivity in lysates of cells treated for the same time with vehicle alone; these control levels ranged from 5,864 ± 338 cpm (12h) to 3,429 ± 224 cpm (0 h). Data are means (± s.e.m.) of two experiments, each performed in duplicate.

Figure 12 shows pharmacokinetic profile of PTHrP(1-36) and [I^5]-PTHrP(1-36) in normal rats. Plasma concentrations of peptides were measured by radioimmunoassay (RIA). The $His^5 \rightarrow Ile$ substitution in PTHrP(1-36) did not significantly change the pharmokinetic profile.

Figures 13A-13C are a set of graphs showing the effects of PTHrP(1-36) and [I^5]-PTHrP(1-36) in normal rats. Figure 13A shows transient calcemic action of PTHrP(1-36) and [I^5]-PTHrP(1-36) in normal rats. The $His^5 \rightarrow Ile$ substitution in PTHrP(1-36), which increased affinity for R^0 by 9-fold (see Table inset) resulted in a more prolonged calcemic effect. Figures 13B and 13C show the delayed (60 min; Figure 13B) and the maximal (Figure 13C) cAMP response in cells treated with each of these ligands.

Figures 14A-14C are graphs showing prolonged calcemic effects in TPTX rats (Figure 14A) and prolonged cAMP signaling in ROS 17/2.8 cells (Figures 14B and 14C) for Mc-PTH(1-14)/PTHrP(15-36) (Mc = $Ala^{1,3,12}$,Gln^{10},Arg^{11},Trp^{14}, Arg^{19}). Figures 14B and 14C show the delayed (60 min; Figure 14B) and the maximal (Figure 14C) cAMP response in cells treated with hPTH(1-34) or Mc-hPTH(1-14)/PTHrP(15-36). The Table inset shows binding affinities for the analogs at the R^0 and RG receptor conformations, measured in vitro.

Figures 15A and **15B** are graphs showing transient calcemic action of modified PTH/PTHrP hybrids in normal rats. Prolonged calcemic effects are

observed for Mc-PTH(1-11)/PTHrP(15-36) and Mc-PTH(1-14)/PTHrP(15-36). The Table inset shows binding affinities for the analogs at the R^0 and RG receptor conformations, measured in vitro.

Figures 16A-16C are graphs showing calcemic action of Mc-modified PTH(1-34) analogs with or without the $Ile^5 \rightarrow His$ and $Arg^{19} \rightarrow Glu$ substitutions, in normal rats (Figure 16A) and delayed and maximal cAMP responses in ROS 17/2.8 cells (Figures 16B and 16C). The Table inset shows binding affinities for the analogs at the R^0 and RG receptor conformations, measured in vitro. The $Ile^5 \rightarrow His$ and $Arg^{19} \rightarrow Glu$ substitutions reduce affinity for R^0, and reduce duration of cAMP signaling in vitro and the calcemic effect in vivo.

Figures 17A-17C are graphs showing transient calcemic action of Mc-modified PTH(1-34)/PTHrP(1-36) analogs without the $Ile^5 \rightarrow His$ and $Arg^{19} \rightarrow Glu$ substitutions in normal rats and delayed cAMP and maximal response in ROS 17/2.8 cells (Figures 17B and 17C). The Table inset shows binding affinities for the analogs at the R^0 and RG receptor conformations, measured in vitro. The $Ile^5 \rightarrow His$ and $Arg^{19} \rightarrow Glu$ substitutions reduce affinity for R^0, and reduce duration of cAMP signaling in vitro and the calcemic effect in vivo.

Figures 18A and **18B** are graphs showing the calcemic and cAMP actions of E^{19},Mc-modified PTH(1-34) analogs, with or without the $Trp^{23} \rightarrow Ala$ substitution in normal rats (Figure 18A) and in ROS 17/2.8 cells (Figure 18B). The Table inset shows binding affinities for the analogs at the R^0 and RG receptor conformations, measured in vitro. The $Trp^{23} \rightarrow Ala$ substitution reduced binding affinity of [E^{19},Mc]PTH(1-34) for R^0 by 10-fold, reduced duration of cAMP signaling in cells, and reduced the hypercalcemic effect of this peptide in vivo.

Figures 19A and **19B** are graphs showing cAMP signaling of native PTH/PTHrP hybrid analogs in cells expressing the human PTH1 receptor. The analogs show similar potencies in acute dose-response assays.

Figures 20A and **20B** are graphs showing cAMP signaling of Mc-modified PTH/PTHrP hybrid analogs with human PTH1 receptor. The analogs show similar potencies in acute dose-response assays.

Figures 21A and **21B** are graphs showing acute (Figure 21A) and delayed (Figure 21B) cAMP analyses in ROS 17/2.8 cells of hPTH(1-34)NH_2, hPTH(1-28)NH_2 and [A^1,Aib^3,M]-PTH(1-28)NH_2 ([$A^{1,12}$,Aib^3,Q^{10},homoarginine^{11},W^{14},R^{19}]hPTH(1-28)NH_2). In Figure 21A, cells were incubated with peptides in the presence of IBMX for 10 minutes, and cAMP was measured. The EC_{50} values were 0.32, 7.6, and 0.33 nM, respectively. In Figure 21B, the cells were treated with 10^{-7} M of hPTH(1-34), [A^1,Aib^3,M]-PTH(1-28), or 10^{-6} M of hPTH(1-28) for 10 minutes, washed three times, incubated in buffer alone for the times indicated, treated for a final 5 minutes with IBMX, and then cAMP was measured. The data in Figure 21B are expressed as a percent of the maximum response observed for each ligand, determined by incubating the cells with ligand in the presence of IBMX for 10 minutes (no ligand wash-out). These values were 67 ± 6; 68 ± 3; and 71 ± 1 pmole/well, respectively. The basal (vehicle) cAMP value was 3.7 ± 0.4 pmole/well.

Figures 22A-22C are graphs showing pharmacokinetic analysis of PTH ligands injected into mice, assessed by a bioassay procedure using COS-7 cells transfected with the PTHR (Figures 22A and 22C) for activity read-out. COS-7 cells transfected with the pCDNA1 vector were used as controls (Figure 22B). Mice were injected with vehicle, with hPTH(1-34) (50 nmol/kg), hPTH(1-28) (1,000 nmol/kg), or [A^1,Aib^3,M]-PTH(1-28) (50 nmol/kg) and at the indicated times after injection, blood was collected from the tail vein, plasma was prepared in the presence of EDTA and proteinase inhibitors, the plasma was diluted 50-fold, and 45 µl of the diluted sample was applied to COS cells in 96-well plates. Then, following a 15 minute incubation, the intracellular cAMP in the COS cells was measured. Each tracing shows data (mean+SE), from six identically treated mice.

Figure 23 is a graph showing changes in blood ionized calcium in mice. Shown are the changes in blood ionized calcium (iCa^{++}) in mice treated with hPTH(1-34) (50 nmol/kg), hPTH(1-28) (1,000 nmol/kg), or [A^1,Aib^3,M]-PTH(1-28) (50 nmol/kg), at times after injection (studies performed in conjunction with those of Figures 22A-22C). Data are normalized to the iCa^{++} in blood drawn from each mouse prior to injection (pre). Each trace shows data (mean+SE) from six identically treated mice.

Figures 24A and **24B** are graphs showing changes in bone-formation and bone-resorption markers in mice after long-term treatment with PTH ligands. Shown are the serum levels of the bone-formation marker osteocalcin (Figure 24A) and the bone-resorption marker, collagen-type I C-terminal fragment (CTX) (Figure 24B) in mice treated with hPTH(1-34) (50 nmol/kg), and [A^1,Aib^3,]M-PTH(1-28) (50 nmol/kg). Markers were measured using Mouse Osteocalcin EIA kit (Biomedical Technologies) and RatLaps CTX ELISA (Nordic Bioscience) kit. Each trace shows data (mean+SE) from six identically treated mice.

Figure 25 is a table showing cAMP signaling potency of PTH/PTHrP hybrid analogs on the human PTH receptor in HKRK-B7 cells.

Figure 26A is a table showing competition analysis of R^0 and RG binding of PTH/PTHrP analogs with the human PTH receptor expressed in COS-7 cell membranes.

Figure 26B is a table showing the same data as Figure 26A, sorted by R^0 binding values.

Figures 27A-27D are graphs showing alanine-scan and type-substitution of PTHrP(1-28). The effects of alanine substitutions in the 15-28 region of PTHrP(1-28) on cAMP activity was examined in renal tubule LLCPK1-B64 (Figure 27A) and ROS17/2.8 (Figure 27B) cells. Alanine substitution at position 18, 22, 25 and 26 increased activity in at least one cell type. These positions were further substituted to various types of amino acids, and cAMP activity was analyzed in LLCPK1-B64 cells (Figure 27C) or SaOS-2 cells (Figure 27D). Cells were treated with analogs at $3x10^{-9}$ M in the presence of

IBMX for 30 minutes at room temperature. Responses for each analog were normalized to the response for the parent (native) PTHrP(1-28) peptide.

Figures 28A and **28B** are graphs showing cAMP activity in vitro (Figure 28A) and in vivo (Figure 28B) by peptides having substitutions in the PTHrP(1-28) scaffold. Dose response curves of cAMP activity of representative modified PTHrP(1-28) analogs in SaOS cells are shown in (Figure 28A). Figure 28B shows in vivo cAMP induction, from C57BL/6 mice (3 month old, male) injected intravenously with either vehicle, PTHrP(1-36), PTHrP(1-28), $A^{18},^{22},L^{25},K^{26}$ (AALK)-PTHrP(1-28) or $E^{18},A^{22},L^{25},K^{26}$ (EALK)-PTHrP(1-28) (n= 3). Blood was withdrawn 10 minutes after injection, and the plasma level of cAMP was measured by RIA.

Figures 29A and **29B** are graphs showing the effect of R^0 and RG selective PTH analogs on plasma cAMP and calcium in mice. Figures 29A and 29B show plasma cAMP concentrations in mice (C57BL/6, males, 3 months) that were administered either vehicle, rPTH(1-34), M-PTH(1-34) ($M=A^1,Aib^3,Q^{10},Har^{11},A^{12},W^{14},R^{19}$), or $E^{18},A^{22},L^{25},K^{26}$-(EALK)-PTHrP(1-30) (5 nmol/kg; n= 7 for cAMP, n=4 for calcium) intravenously. Figure 29B shows ionic calcium levels in mice treated with the same peptides. In the calcium experiment, blood was withdrawn before, and 1, 2, 4 and 6 hours after injection, and ionized calcium was measured using a Ca^{++}/pH analyzer.

Figures 30A-30F are graphs showing the effects of PTH analogs on plasma bone markers in mice. Mice (C57BL/6, males, 3 months) were intravenously injected daily with either vehicle, rPTH(1-34), M-PTH(1-34), or (EALK)-PTHrP(1-30) (5 nmol/kg; n= 7 group) for 14 days. Markers of bone turnover (PINP, CTX and osteocalcin) were assessed by ELISA in blood at day 6 (Figures 30A, 30C, and 30E, respectively) and 13 (Figures 30B, 30D, and 30F, respectively).

Figure 31 is a set of images showing the effects of two-week daily treatment of R^0 and RG ligands on trabecular and cortical bone structure in mice. Mice (C57BL/6, males, 3 months) were treated (i.v.) with either vehicle,

rPTH(1-34), M-PTH(1-34), or E^{18},A^{22},L^{25},K^{26} (EALK)PTHrP(1-30) (5 nmol/kg; n= 7 group), daily for 14 days, and femurs were analyzed by μCT.

Figures 32A and **32B** are graphs showing the effects of amino acid substitutions in the 29-31 region of EALK-PTHrP(1-31) (Figure 32A) and the 29-33 region of EALK-PTHrP(1-34) (Figure 32B) on induction of cAMP activity in MC3T3-E1 cells.

Figure 33 is a graph showing calcemic action of PTH(1-34) and M-PTH(1-14)/PTHrP(15-36) (SP-PTH) in TPTX rats from time zero to 24 hours.

Figure 34 is a graph showing urinary calcium at 0-6 hours following a single injection of SP-PTH or PTH(1-34) in TPTX rats.

Figure 35 is graph showing hypophosphatemic action of PTH(1-34) and SP-PTH in TPTX rats.

Figure 36 is a graph showing urinary phosphorus at 0-6 hours after a single injection of SP-PTH or PTH(1-34) in TPTX rats.

Figure 37 is a graph showing a dose-response analysis of cAMP signaling potency for Mc-PTH(1-34), [$A^{1,3}$,A^{23},Q^{10},R^{11}]-hPTH(1-34), [$A^{1,3}$,A^{23}]-hPTH(1-34), and [A^{18},A^{22},L^{25},K^{26}]-PTHrP(1-28). For comparison, hPTH(1-34) and PTHrP(1-36) are also shown. The capacity of these peptides to stimulate cAMP formation was assessed on the human PTH1 receptor in HKRK-B7 cells. These PTH analogs show comparable cAMP signaling to hPTH(1-34).

Detailed Description

We have discovered a correlation between (i) the ability of a GPCR ligand to bind a GPCR when uncoupled to a G-protein (the R^0 state) and (ii) the length of time over which the ligand activates the receptor. In particular, an enhanced ability of a ligand to interact in vitro with the exemplary GPCR, the PTH/PTHrP receptor (PTHR), uncoupled to a G-protein (the R^0 form), as compared to PTH or PTHrP, closely correlates its ability to exert more prolonged activity in vivo. The reverse is also true, i.e., that ligands selective for the G-protein coupled forms of GPCR (the RG form) have a shorter

duration of activity as compared to the native ligand. This discovery provides the basis for a novel means of determining whether a compound has either long-acting or shorting-acting in vivo activity on a GPCR. On this basis, ligands with therapeutically desirable properties (e.g., long-acting or short-acting ligands) can be identified using the methods described herein. Examplary ligands with either long-acting or short-acting activity are described herein.

Depending on the disease being treated, long-acting or short-acting therapeutics are desirable. Recent studies using PTHrP(1-36) injected in humans show that bone mineral density increased to about the same extent as with PTH(1-34), the standard therapy for osteoporosis, but without inducing the bone-resorptive responses that would be expected for an equivalent dose of PTH(1-34) (Horwitz et al., *J. Endocrinol. Metab.* 88:569-575 (2003)). Related studies from this group suggest that the differences are not likely based solely on pharmacokinetics, as an acute safety study indicated that PTHrP(1-36) could be administered at doses nearly 20-fold above the usual dose of PTH(1-34) without producing a hypercalcemic effect (Horwitz et al., *Osteoporosis Int.* 17:225-230 (2006)). While both PTHrP(1-36) and PTH(1-34) exhibit similar receptor binding to the RG form of the PTHR, our discovery that PTHrP binds less strongly to the R^0 form of the PTHR and correspondingly exhibits less prolonged activity in vivo as compared to PTH can explain the difference. Accordingly, we believe that RG selective ligands of PTHR (i.e., with relatively low R^0 affinity) will prove useful for treatment of osteoporosis.

In other situations, a longer acting ligand may be desirable. For example, PTHrP is less effective than PTH(1-34) in stimulating renal production of 1,25,$(OH)_2$vitamin D (Horwitz et al., *J. Bone Mineral. Res.* 20:1792-1803 (2005)), suggesting that PTH(1-34) may be more effecting in treating disease where long-acting PTHR signaling is desired. Such diseases include certain forms of hypoparathyroidism caused by activating mutations in the calcium-sensing receptor. Currently, treating this disease requires twice daily injections of PTH(1-34) (Winer et al., *J. Clin. Endocrinol. Metab.*

88:4214-4220 (2003)). By using the screening methods of the invention, it becomes possible to identify longer acting PTHR ligands, which can prove highly useful in the treatment of such diseases and may allow for less frequent administration of the drug.

PTH(1-34), via its greater capacity to bind stably to R^0, may be able to induce a cumulatively greater signaling response in target bone and kidney cells than does PTHrP, and this difference in R^0 selectivity then leads to a divergence in biological responses, such as the induction in osteoblasts of factors (RANK Ligand) involved in stimulating osteoclastic bone resorption, and the stimulation in renal proximal tubule cells of 1-α-hydroxylase mRNA synthesis. According to these considerations, a ligand that binds with particularly high selectivity to the RG (versus R^0) PTHR conformation might be highly effective in stimulating bone formation responses, and thus useful for treating osteoporosis.

Thus, the two ligands preferentially stabilize distinct receptor conformations. There is now much discussion in the GPCR field regarding the capacity of structurally varied ligands for a given receptor to exhibit altered selectivities for distinct receptor conformations, and thus produce distinct biological effects (Kenakin, T. *Sci STKE* 342:pe29 (2006)). The results of the kinetic and equilibrium binding assays performed herein suggest that whereas PTH(1-34) and PTHrP(1-36) bind with similar affinities to the G protein-coupled PTHR conformation, RG, PTH(1-34) exhibits a greater capacity to bind to the G protein-uncoupled conformation, R^0, defined as a receptor conformation that has the capacity to bind ligand with high affinity in the presence in GTPγS (5,14), than does PTHrP(1-36).

The delayed cAMP assays presented herein demonstrate that altered selectivity for distinct PTHR conformations can lead to altered signaling responses in PTHR-expressing cells. Thus, PTH(1-34) and Ile^5-PTHrP(1-36) induced more prolonged, and cumulatively greater, cAMP signaling responses in PTHR-expressing cells. PTH(1-34) and Ile^5-PTHrP(1-36), which also have a greater capacity to stabilize R^0 than PTHrP(1-36), can induce more prolonged

signaling responses due to the eventual coupling of the LR^0 complex to a heterotrimeric G protein (LR^0 - LRG) and activation of the corresponding signaling cascade. Another potential mechanistic consequence of stable LR^0 binding is that it may permit multiple (catalytic) rounds of G protein activation, by which an LR^0 complex is preserved after successive cycles of G protein coupling, activation and release (Rodbel, M. *Adv. Enzyme Regul*, 37: 427-435 (1997); Heck and Hofmann, *J. Biol. Chem.* 276:10000-10009 (2001)).

Little if any difference in the potencies with which PTH(1-34) and PTHrP(1-36) ligands stimulated cAMP and inositol phosphate responses was detected when the ligands were assessed in conventional dose-response, cAMP and inositol phosphate stimulation assays performed in cells at a single-time-point (Figure 7). These results are consistent with the view that the two ligands interact with the PTHR via the same, or similar mechanisms. The time-delayed cAMP assays thus identified previously unappreciated differences in the second-messenger signaling properties of the two ligands, evident as differences in the cumulative signal output over time. While the agonist-activated PTHR is known to be subject to desensitization processes involving receptor phosphorylation, beta-arrestin recruitment, and receptor internalization (Biselo, A. et al., (2002); Tawfeek et al., *Mol. Endocrinol.* (2002); Castro et al., *Endocrinology* 143:3854-3865 (2002); Chauvin et al., *Mol. Endocrinol.* 16:2720-2732 (2002)), it is not expected that such a process would operate on receptors in the R^0 conformation, as these are, by definition, functionally inactive, at least in terms of G protein coupling. Nevertheless, the possibility that the effects observed in our delayed cAMP assays of Figure 5 involve, to some extent, differential effects of the ligands on such receptor desensitization mechanisms cannot be excluded.

In general, a stable LR^0 binding capacity might facilitate, or augment, the signaling potential of a ligand in target cells that express a low level of the cognate heterotrimeric G protein, relative to the target receptor. It may also facilitate coupling to "secondary" G proteins that presumably have lower affinity for the ligand-receptor complex than does the primary G protein.

For the PTHR, this could involve coupling to $G\alpha_{q/11}$, $G\alpha_{i/o}$, or $G\alpha_{12/13}$, each of which has been shown to be activated by the PTHR in response to PTH(1-34). While PTHrP has at least some capacity to bind R^0 (Figures 3A-3D) and activate delayed cAMP signaling (Figures 5A and 5B), the binding is less than that of PTH(1-34). Indeed, some capacity to form a stable LR^0 complex may be an intrinsic property of the class B GPCRs, as several of these, including the receptors for calcitonin (Hilton et al., *J. Endocrinol.* 166:213-226 (2002)), corticortropin-releasing hormone (Hoare et al., *Peptides* 24:1881-1897 (2003)) and glucagon (Post et al., *J. Biol. Chem.* 267:25776-25785 (1992)) have been shown to form a stable complex with their cognate peptide ligand in the presence of a non-hydrolyzable guanine nucleotide analog.

The findings described herein may also relate to the mechanisms by which PTH and PTHrP function in normal physiology. PTH, as an endocrine hormone, acts on target cells (in bone and kidney) that are distal from its site of secretion (the parathyroid glands). Concentrations of PTH in the serum, while varying marginally as Ca^{++} levels fluctuate, generally stay within the low picomolar range, well below the affinity with which PTH binds to its receptor. The capacity of PTH to bind stably to the receptor even in the uncoupled, R^0 conformation may be an evolutionary adaptation that helps to ensure a response to even minimal increases in the ligand's concentration. By contrast, PTHrP, as a paracrine factor, acts on cells within the same tissue in which it is produced (e.g., the growth-plate chondrocytes of developing long bones). The concentrations of PTHrP in such tissues have not been directly quantified, but they appear to form a gradient across the zones of differentiating cells and high near the sites of production (Chen et al., *J. Bone Miner. Res.* 21:113-123 (2006)). It may be that, as an adaptation for its role in controlling the differentiation events that occur in these cells, PTHrP evolved to bind to the receptor only transiently, so as to induce a relatively short-lived, and more easily timed, signaling response.

G-protein-coupled receptors

The present invention can use any G-protein-coupled receptor. Long-acting and short-lived ligands may be assayed as described herein and useful therapeutic candidates identified. Hundreds of such receptors are known in the art; see, e.g., Fredriksson et al., *Mol. Pharmacol.* 63:1256-1272, 2003, which is hereby incorporated by reference. This reference has characterized the human GPCRs based on sequence homology and function. Human GPCRs can be broken down into five classes: secretin, rhodopsin, glutamate, frizzled/Tas2, and adhesion. Alternatively, receptors may be classified by their ligands, e.g., peptide hormones or small molecules (e.g., biogenic amines). Other classification schemes include the A-F classification, where class A represents receptors related to rhodopsin and the adrenergic receptors, class B, receptors related to the calcitonin and parathyroid hormone receptors, class C, receptors related to the metabotropic receptors, and classes D-F represent receptors found in fungi and archaebacteria.

Using the Fredriksson classification, the secretin receptors have four main subgroups: the CRHRs/CALCRLs, the PTHRs, GLPRs/GCGR/GIPR and the subgroup including secretin and four other receptors. Secretin receptors include the PTHR, as well as the calcitonin receptor (CALCR), the corticotropin-releasing hormone receptors (CRHRs), the glucagon receptor (GCGR), the gastric inhibitory polypeptide receptor (GIPR), the glucagon-like peptide receptors (GLPRs), the growth hormone-releasing hormone receptor (GHRHR), pituitary adenylyl cyclase-activating protein (PACAP), the secretin receptor (SCTR), and vasoactive intestinal peptide receptor (VIPR).

The adhesion receptors feature GPCR-like transmembrane-spanning regions fused together with one or several functional domains with adhesion-like motifs in the N terminus, such as EGF-like repeats, mucin-like regions, and conserved cysteine-rich motifs. Members of this family include the CELSRs (EGF LAG seven-pass G-type receptors), the brain-specific angiogenesis-inhibitory receptors (BAIs), the lectomedin receptors (LECs) and the EGF-like module containing (EMRs). Other receptors include the CD97

antigen receptor (CD97) and EGF-TMVII-latrophilin-related (ETL). These receptors also include HE6 (TMVIILN2) and GPR56 (TMVIIXN1 or TMVIILN4) and a group of recently discovered receptors, related to GPR56 and HE6, named GPR97 and GPR110 to GPR116.

The glutamate receptors consists of eight metabotropic glutamate receptors (GRM), two GABA receptors (e.g., GAB-AbR1, which has two splice variants, a and b, and GAB-AbR2), a single calcium-sensing receptor (CASR), and five receptors believed to be taste receptors (TAS1).

Other GPCRs include opioid, muscarinic, dopamine, adrenergic, cAMP, opsins, angiotensin, serotonin, thyrotropin, gonadotropin, substance-K, substance-P and substance-R, and melanocortin, metabotropic glutamate receptors.

The largest group is the rhodopsin receptor family, which includes at least 701 human receptors, 241 of which are non-olfactory. Receptors in this group include various acetylcholine (muscarinic) receptors, adrenergic receptors, dopamine receptors, histamine receptors, serotonin receptors, and octopamine receptors; peptide receptors, e.g., angiotensin, bombesin, bradykinin, endothelin, interleukin-8, chemokine, melanocortin, neuropeptide Y, neurotensin, opioid, somatostatin, tachykinin, thrombin, vasopressin, galanin, proteinase-activated, orexin, and chemokine/chemotatic factor receptors; protein hormone receptors, e.g., FSH, lutropin-choriogonadotropic hormone, and thyrotropin receptors; rhodopsin receptors; olfactory receptors; prostanoid receptors; nucleotide-like receptors, including adenosine and purinoceptors; cannabis receptors; platelet activating factor receptor; gonadotropin-releasing hormone receptor; melatonin receptor, lysosphingolipid and LPA (EDG) receptors, as well as various orphan receptors.

Candidate compounds

Any type or source of compound may be used in the screening methods of the invention. For example, naturally occurring chemicals (e.g., from a chemical library), peptides, modified peptide hormones, antibodies,

nanobodies, chimeric peptides, and fragments of endogenous ligands (e.g., peptide ligands) may all be used in the present invention. Approaches involving random screening, such as natural libraries of compounds, or designed ligands (e.g., ligands based on the PTH sequence) may be used in the screening methods of the invention. In some embodiments, antibodies or nanobodies can be generated against the GPCR or a ligand binding fragment of the GPRC using methods known in the art.

Modified receptor agonists

One strategy for identification of new receptor agonists is the modification of existing agonists. Peptide hormones can be modified by point mutations, truncations, insertions, and generation of chimeric peptides. Using the PTH receptor, for example, many modified PTH and PTHrP sequences are known in the art. Peptides can made either recombinantly or synthetically, as is known in the art. See, for example, U.S. Patent Nos. 7,057,012, 7,022,815, 6,417,333, 6,495,662, hereby incorporated by reference, which describe various PTH sequences, as well as any of those described herein. These sequences can include chimeric peptides. In one particular example, any agonist may be fused to an antibody or antibody fragment (such as an Fc fragment) to generate a candidate therapeutic.

Antibodies and nanobodies

Antibodies or nanobodies which bind the GPCR can also be used in the methods of the invention and can be raised against the GPCR or a fragment thereof (e.g., a ligand-binding portion of the GPCR) using any method known in the art. In one example, an IgG directed to a GPCR or fragment thereof can be generated in New Zealand white rabbits using a purified protein. The initial immunization protocol consists of an initial intramuscular injection of 10-20 μg purified protein, followed by a boosting immunization 21 days later. Further boosts and/or the addition of adjuvant may be used if no or few antibodies are detected. Antibodies may be quantified by ELISA, analogous to that described

(Siber et al., J. Infect. Dis. 152:954-964, 1985; Warren et al., J. Infect. Dis. 163:1256-1266, 1991). IgG may be purified from the rabbit antiserum, for example, by precipitation in 50% ammonium sulfate followed by affinity chromatography on Protein G sepharose 4B (Pharmacia). Monoclonal antibodies to GPCRs can be produced using hybridoma technology. Nanobodies can be generated by immunization of an animal (e.g., a camel or llama) which produce nanobodies, which can then be purified using standard techniques. These antibodies or nanobodies would be screened as described herein for those agonistic molecules that produce long-lived or short-acting effects.

Test compounds and extracts

In general, compounds capable of binding a GPCR (e.g., PTHR) are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and polynucleotide-based compounds. Synthetic compound libraries are commercially available. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available. In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and

fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their activity in treating metabolic disorders should be employed whenever possible.

When a crude extract is found to bind the GPCR in its RG state, and either exhibits altered binding (e.g., higher affinity or lower affinity) as compared to the endogenous ligand when the receptor is in its R^0 state, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the characterization and identification of a chemical entity within the crude extract having activity that may be useful in treating a metabolic disorder (e.g., diabetes and obesity). Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents in the screening methods of the invention are chemically modified according to methods known in the art.

Such test compounds include naturally occurring or synthetic chemical compounds, (including small molecules) as well as amino acid or nucleic acid aptamers. Any of these compounds may include synthetic or modified amino acids or nucleic acids.

Contacting a receptor with a candidate compound

In the screening method of the present invention, a candidate compound is contacted with a GPCR. The receptor may be found on a cell (e.g., in an organism), or a in a membrane preparation. Alternatively, the receptor may be isolated in functional form (Shimada et al., *J. Biol. Chem.* 277:31,774-31780, 2002).

Cells which either naturally express the GPCR of interest (e.g., PTHR) or express the receptor recombinantly can be used in the methods of the invention. Alternatively, or in addition, the cells can be tranfected (e.g., using any method known in the art) to express a recombinant gene encoding the GPCR. Cells expressing a particular GPCR can also be obtained commercially, for example, from Millipore (ChemiScreen™ cell lines).

In other embodiments, the receptor is present in a membrane preparation (e.g., cell free) which contains the GPCR of interest. Such preparations are commercially available; see, e.g., the ChemiSCREEN™ receptor preparations available from Millipore. Membrane preparations can also be produced using methods known in the art (see, e.g., Mills et al., *J. Biol. Chem.* 263:13-16, 1988).

If purified receptor components are utilized, candidate compound are contacted with the receptor or receptor complex in vitro.

Assay readout - measuring ligand binding or activity

Any method for analysis of ligand binding or ligand activity may be used in the methods of the invention; the particular readout is not critical. In some embodiments, ligand binding to the GPCR is measured by displacement of a radiolabeled ligand by a non-labeled compound and measuring the radioactivity of the cell or membrane preparation before and after treatment with the non-labeled compound. In general, this approach involves incubating the membranes and radioligand to allow complex formation. Dissociation phase can be initiated by the addition of excess unlabeled compound. Immediately prior to the addition (t=0), and at successive time-points thereafter, aliquots can be withdrawn and immediately processed by vacuum filtration. Non-specific binding is determined in parallel reaction tubes containing the unlabeled compound in both the pre-incubation and dissociation phases. The specifically bound radioactivity at each time point can be calculated as a percent of the radioactivity specifically bound at t=0.

Such dissociation methods are well suited to large scale screening (e.g., libraries of candidate compounds).

As described in Example 1 below, other methods such as FRET can also be used to measure ligand binding to a receptor. In one application, two fluorescent molecules are conjugated to the receptor such that ligand binding results in a conformational change in the receptor that can be detected by a change in FRET signal. FRET allows for real time measurement of ligand binding and is thus useful in the assays of the invention.

Other readouts include measurements of cAMP activity including the delayed cAMP activity assay described herein, which indirectly measures binding of the compound to the RG form of the receptor. Intracellular cAMP levels can be measured using a radioimmuno assay, e.g., as described by Shimizu et al. (*J. Biol. Chem.* 276:49003-49012 (2001)). Briefly, this method includes treatment with a candidate compound, rinsing with 0.5 ml of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.7 with HCl), and treating with 200 µl of cAMP assay buffer (Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 µl of binding buffer containing varying amounts of the candidate compouond (final volume = 300 µl). The medium can then be removed after incubation for 30-60 min at room temperature. The cells can then be frozen, lysed with 0.5 ml 50 mM HCl, and refrozen (at -80 °C). The cAMP content of the diluted lysate can be determined by radioimmunoassay. The EC_{50} response values can be calculated using nonlinear regression.

Any suitable physiological change that affects GPCR activity can be used to assess the influence of a test compound on GPCR activity. When the functional consequences are determined using intact cells or animals, a variety of effects such as transmitter release, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in

intracellular second messengers such as Ca^{++}, IP_3, or cAMP, can also be measured.

In one embodiment, the changes in intracellular cAMP can be measured using immunoassays. The method described in Offermanns and Simon, *J. Biol. Chem.* 270:15175-15180 (1995), may be used to determine the level of cAMP. Assay kits for measuring cAMP as described in U.S. Pat. No. 4,115,538, herein incorporated by reference, can also be used. Other assays that may be used include measuring in vivo changes in serum/urinary calcium, phosphate, and markers of bone-turnover (e.g., deoxypridonoline crosslinks), decreases in serum reciprocal changes in urine.

Measuring R^0 or RG binding

The methods of the present invention involve measurement of binding of a candidate compound to the RG or R^0 form of the GPCR (e.g., PTHR). Thus, the readout of the assay can distinguish between the affinity of the compound for each form of the receptor. One possible approach is to use a system or condition where one receptor conformation is favored. R^0 can be favored, for example, by forced dissociation of the GPCR from its G-protein, or using a system that lacks G-proteins. One manner in which dissociation of the GPCR from G-proteins can be achieved is by treatment with a compound that prevents binding of the G-protein to its GPCR. Such compounds include nucleotide analogs such non-hydrolyzable nucleotide analogs including GTPγS. GTPγS binds the G-protein, but as it is unable to hydrolize this compound, the G-protein cannot recycle itself back on the GPCR. Thus, by contacting a cell or cell membrane with GTPγS prior to addition of the candidate compound, it is possible to generate a system in which the R^0 state of the GPCRs is highly favored.

To stabilize the RG form of the GPCR, dominant-negative G-proteins can be used. These proteins bind the GPCR in a stable manner, and thus enrich for the RG conformation.

Other approaches to modulate the ratio between R^0 and RG include using cells from animals in which expression of one or more G-proteins has been downregulated or eliminated. Genetic knockout technologies are well known in the art and can be used to target specific G-proteins (see, e.g., Dean et al., Mol. Endocrinol. 20:931-943 (2006)). In other embodiments, RNAi techniques (e.g., administration of siRNA to a cell) can be used to "knock down" expression of G-proteins, thereby favoring the R^0 state of the receptor. Alternatively, it may be possible to favor the RG form by overexpressing the appropriate G protein or G-proteins in a cell.

A second approach for measuring the ability of a compound to bind either the R^0 or RG state involves displacement of a ligand known to be selective for a particular state. In the case of the PTH receptor, previous work has shown that ^{125}I-[Aib^{1,3},M]PTH(1-15) is selective for the RG state. By measuring ligand displacement by a candidate compound of a such ligand, the binding of the compound to that state can be specifically measured, even if the receptor is present in both the RG and the R^0 states in the assay.

Compounds identified in the methods of the invention typically bind to the RG form of the receptor with at least 5%, (e.g., at least 10%, 20%, 50%, 100%, 500%, 1000%, 10,000%) of the activity of an endogenous receptor for either long-acting or short-lived agonists. For example, human PTH binds the human PTHR with an EC50 of about 0.13 nmol. Thus desirable compounds typically bind the hPTHR with at least 10% of this affinity, i.e., at least 1.3 nmol EC50.

Ligands identified using the methods of the invention

Using the screening methods described herein, we have identified a variety of ligands for the exemplary GPCR, the PTH receptor, representing different combinations of either class of peptide (PTH/PTHrP hybrids) chosen on the basis of their relative R^0/RG selectivity to be either short-acting ligands or long-acting ligands (Figures 26A and 26B). Based on the results of our screening assay, we then tested these peptides for in vitro and in vivo activity

to demonstrate proof of concept of the importance of R^0/RG selectivity in determining biological activity of the ligand.

The identified peptides represent proof of concept for the PTH receptor and other GPCRs that R^0/RG selectivity determines biological action in vivo. These peptides include five different classes. A first class is typified by Ile^5-PTHrP, an analog that converts PTHrP to a form with high R^0 selectivity and prolonged action. A second class includes hybrid peptides with high R^0/RG selectivity composed of MPTH(1-11) combined with PTHrP(12-36) or MPTH(1-14) with PTHrP(15-36). These peptides have very prolonged biological activity in vivo. The third type is [His^5,Arg^{19}]PTH, which illustrates shorter acting biological activity due to its reduced R^0 affinity. A fourth class of compounds is exemplified by Ala^1,Aib^3-M-PTH(1-28), which has a potent R^0-activating activity, as well as striking activity to promote urinary phosphate excretion, a property desirable in the treatment of disorders associated with high phosphate retention. A fifth class is typified by Ala^{23}-PTH, which has a much lower R^0 affinity and therefore more desirable for the treatment of osteoporosis.

For the PTH receptor ligands, we have identified ligands with variety of R^0 and RG binding affinities and various R^0/RG selectivities. Exemplary peptides, sorted by R^0 affinity are shown in Figure 26B. The affinity for the R^0 form of the receptor may be at least 2000, 1000, 750, 500, 250, 150, 100, 90, 75, 50, 40, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.5, 0.2, 0.1, or 0.05 nmol. The affinity for the RG form of the receptor may be at least 100, 50, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2.5, 2, 1.75, 1.5, .125, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15, 0.1, 0.075, 0.05, 0.025 nmol. The selectivity of R^0/RG may be (where a higher values indicates greater RG-selectivity) at least 0.5, 1, 2, 3, 4, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 75, 100, 150, 200, 250, 400, 500, 750, 1000, 1250, 1500, 2000, 2500, or 5000. Ligands of the invention may have any of the RG or R^0 affinities described herein, or any combination thereof.

RG and R^0 selective ligands

Using the screening methods described herein, we have developed new RG selective and R^0 selective ligands. In one example, we used PTHrP(1-28) as a starting point, as PTHrP binds to the RG receptor conformation with greater selectivity as compared to PTH. Table 2 summarizes the in vitro activities of particular analogs; additional analogs are shown in Table 3. More detailed information regarding these analogs are described below in Example 3. These analogs, A(E)18, A22, (L25), K26-PTHrP(1-28) or (1-30) generally exhibit enhanced potency for cAMP generation, and bind with relatively high selectivity to the RG conformation, as compared to PTHrP(1-36) (Table 2).

Table 2. In vitro activities of representative PTHrP analogs

Analog	SaOS cAMP EC50 (nM)	MC3T3-E1 cAMP EC50 (nM)	RG binding affinity hPTHR IC50 (nM)	R^0 binding affinity hPTHR IC50 (nM)	R0/R selecti
PTHrP(1-36)	0.190	0.322	0.33	74.8	22
PTHrP(1-28)	20.3	4.09	0.66	20449	310
A18,22,K26-PTHrP(1-28)	0.024	0.091	0.10	1815	180
E18,A22,K26-PTHrP(1-28)	0.241	0.251	0.24	9237	383
A18,22,L25,K26-PTHrP(1-28)	0.002	0.054	0.04	310	697
E18,A22,L25,K26-PTHrP(1-28)	0.010	0.083	0.10	1741	183
A18,22,L25,K26-PTHrP(1-30)	0.008	0.067	0.05	144	302
E18,A22,L25,K26-PTHrP(1-30)	0.063	0.059	0.08	945	111

Additional peptides and binding/activity data for such peptides are shown in Table 3 below.

Table 3: Binding/activity of PTHrP analogs

	screen			dose-response					
Sequence (parent shown in bold)	cAMP (% parent)[1]	human PIR RG (% parent)[2]	human PIR R0 (% parent)[2]	cAMP in SaOS (EC50, nM)	cAMP in MC3T3-E1 (EC50, nM)	human PIR RG (IC50 nM)	human PIR R0 (IC50 nM)	rat PIR RG (IC50 nM)	rat PIR R0 (IC50 nM)
PTHrP(1-28)NH									
A18-PTHrP(1-28)NH	164								
S18-PTHrP(1-28)NH	121								
M18-PTHrP(1-28)NH	113								
F18-PTHrP(1-28)NH	109								
E18-PTHrP(1-28)NH	140								
A22-PTHrP(1-28)NH	185								
S22-PTHrP(1-28)NH	141								
L22-PTHrP(1-28)NH	142								

N22-PTHrP(1-28)NH	138								
W22-PTHrP(1-28)NH	129								
E22-PTHrP(1-28)NH	121								
K22-PTHrP(1-28)NH	150								
A26-PTHrP(1-28)NH	142								
S26-PTHrP(1-28)NH	107								
N26-PTHrP(1-28)NH	113								
K26-PTHrP(1-28)NH	142								
R26-PTHrP(1-28)NH	143								
L25-PTHrP(1-28)NH	325								
W25-PTHrP(1-28)NH	270								
K25-PTHrP(1-28)NH	163								
R25-PTHrP(1-28)NH	204								
A18,22,26-PTHrP(1-28)NH	343	167	160						
A18,22,K26-PTHrP(1-28)NH	405	193	178	0.024	0.091	0.10	1815		
A18,26,S22-PTHrP(1-28)NH	229	148	133						
A18,S22,K26-PTHrP(1-28)NH	372	175	155	0.038					
A18,26,N22-PTHrP(1-28)NH	265	161	136						
A18,N22,K26-PTHrP(1-28)NH	326	172	139						
A18,26,L22-PTHrP(1-28)NH	252	163	133						
A18,L22,K26-PTHrP(1-28)NH	350	177	160						
A18,26,W22-PTHrP(1-28)NH	188	120	126						
A18,W22,K26-PTHrP(1-28)NH	267	115	136						
E18,A22,K26-PTHrP(1-28)NH	301	145	68.8	0.241	0.251	0.24	9237		
E18,S22,A26-PTHrP(1-28)NH	119	132	31.9						
E18,N22,A26-PTHrP(1-28)NH	171	140	53.7						
E18,N22,K26-PTHrP(1-28)NH	236	147	84.4						
E18,L22,A26-PTHrP(1-28)NH	139	125	52.5						
E18,L22,K26-PTHrP(1-28)NH	264	152	64.4						
E18,W22,A26-PTHrP(1-28)NH	75	116	18.8						
E18,W22,K26-PTHrP(1-28)NH	165	149	46.6						
E18,K22,A26-PTHrP(1-28)NH	315	192	106.1						
E18,K22,26-PTHrP(1-28)NH	374	208	119.8						
E18,A22,26-PTHrP(1-28)NH	190								
A18,22,L25,K26-PTHrP(1-28)NH	305			0.002	0.054	0.04	310	0.16	34.9
A18,22,K25,26-PTHrP(1-28)NH	349			0.012					
A18,22,I25,K26-PTHrP(1-28)NH	342								
A18,22,W25,K26-PTHrP(1-28)NH	329								
A18,22,F25,K26-PTHrP(1-28)NH	337								
A18,S22,L25,K26-PTHrP(1-28)NH	367			0.009		0.10	540		
A18,S22,K25,26-PTHrP(1-28)NH	316			0.015					
E18,A22,L25,K26-PTHrP(1-28)NH	340			0.010		0.10	1741		
E18,A22,K25,26-PTHrP(1-28)NH	323			0.054					
E18,S22,L25,K26-PTHrP(1-28)NH	337			0.055		0.11	2056		
E18,S22,K25,26-PTHrP(1-28)NH	335								
PTHrP(1-30)NH									
A18,22,K26-PTHrP(1-30)NH					0.058				
E18,A22,K27-PTHrP(1-30)NH					0.082				
A18,22,L25,K26-PTHrP(1-30)NH					0.067	0.05	144	0.13	11.1
E18,A22,L25,K26-PTHrP(1-30)NH					0.059	0.08	945	0.21	76.3
PTHrP(1-31)NH									
A18,22,K26-PTHrP(1-31)NH					0.060				
E18,A22,K27-PTHrP(1-31)NH					0.060			0.23	54.8
A18,22,L25,K26-PTHrP(1-31)NH					0.20				
E18,A22,L25,K26-PTHrP(1-31)NH					0.112				
E18,A22,L25,K26-PTHrP(1-31)OH	**100**				**0.78**				
E18,A22,L25,K26,G29-PTHrP(1-31)OH	206								
E18,A22,L25,K26,S29-PTHrP(1-31)OH	209				0.41				

E18,A22,L25,K26,N29-PTHrP(1-31)OH	210	
E18,A22,L25,K26,Q29-PTHrP(1-31)OH	226	0.59
E18,A22,L25,K26,W29-PTHrP(1-31)OH	142	
E18,A22,L25,K26,E29-PTHrP(1-31)OH	100	
E18,A22,L25,K26,K29-PTHrP(1-31)OH	227	0.28
E18,A22,L25,K26,G30-PTHrP(1-31)OH	286	
E18,A22,L25,K26,S30-PTHrP(1-31)OH	331	0.12
E18,A22,L25,K26,L30-PTHrP(1-31)OH	185	
E18,A22,L25,K26,N30-PTHrP(1-31)OH	189	
E18,A22,L25,K26,D30-PTHrP(1-31)OH	251	0.32
E18,A22,L25,K26,K30-PTHrP(1-31)OH	245	0.20
E18,A22,L25,K26,S31-PTHrP(1-31)OH	99	
E18,A22,L25,K26,L31-PTHrP(1-31)OH	198	0.25
E18,A22,L25,K26,V31-PTHrP(1-31)OH	181	
E18,A22,L25,K26,K31-PTHrP(1-31)OH	134	
E18,A22,L25,K26-PTHrP(1-34)OH	**100**	**0.45**
E18,A22,L25,K26,A30-PTHrP(1-34)OH	237	0.14
E18,A22,L25,K26,A31-PTHrP(1-34)OH	249	0.15
E18,A22,L25,K26,A32-PTHrP(1-34)OH	197	
E18,A22,L25,K26,A33-PTHrP(1-34)OH	196	
E18,A22,L25,K26,Q29,D30,V31, N33,F34-PTHrP(1-34)OH	204	0.56

We also produced the peptides A^{20},Mc-PTH(1-34)OH, F^{23},Mc-PTH(1-34)OH, [A^1,A^3,A^{23},Q^{10},R^{11}]-PTH(1-34)OH, [A^1,A^3,A^{23}]-PTH(1-34)OH, and E^{18},A^{22},L^{25},K^{26}-PTHrP(1-30). R^0 and RG binding of these peptides to the human PTH1 receptor is shown in Table 4 below.

Table 4. RG and R^0 binding of exemplary peptides

Peptide	R0 binding IC50 (nM)	RG binding IC50 (nM)	R0/RG ratio
hPTH(1-34)	8.7 ± 1.2	0.13 ± 0.02	67
hPTHrP(1-36)	37.7 ± 4.7	0.14 ± 0.02	260
A20,Mc-PTH(1-34)OH	31.9 ± 10.5	0.40 ± 0.09	80
F23,Mc-PTH(1-34)OH	1.2 ± 0.4	0.23 ± 0.07	5
[A1,3,23,Q10,R11]-PTH(1-34)OH	197 ± 33	0.14 ± 0.00	1407
[A1,3,23]-PTH(1-34)OH	1845 ± 170	0.43 ± 0.09	4291
E18,A22,L25,K26-PTHrP(1-30)	945.0 ±	0.08 ±	11813

Mc=A1,3,12,Q10,R11,W14,R19

Polypeptide modifications

Any of the polypeptides described herein may contain one or more modifications such as N-terminal or C-terminal modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a

lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, garnma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as aiginylation, and ubiquitination. See, for instance, Proteins-Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al, Methods Enzymol 182:626 646 (1990) and Rattan et al, Ann NY Acad Sci 663A& 62 (1992).

Any of the polypeptides of the invention may further include a heterologous sequence (a fusion partner), thus forming a fusion protein. The fusion protein may include a fusion partner such as a purification or detection tag, for example, proteins that may be detected directly or indirectly such as green fluorescent protein, hemagglutinin, or alkaline phosphatase), DNA binding domains (for example, GAL4 or LexA), gene activation domains (for example, GAL4 or VP16), purification tags, or secretion signal peptides (e.g., preprotyrypsin signal sequence). In other embodiments the fusion partner may be a tag, such as c-myc, poly histidine, or FLAG. Each fusion partner may contain one or more domains, e.g., a preprotrypsin signal sequence and FLAG tag. In other cases, the fusion partner is an Fc protein (e.g., mouse Fc or human Fc).

Methods of treatment of disease

Any disease associated with PTH dysfunction, or calcium or phosphate imbalances, can be treated with any of the peptides described herein, including those in Figures 26A and 26B, those of Table 1, or those identified using the

methods of the invention. The peptides may be used to treat osteoporosis, fracture repair, osteomalacia, arthritis, thrombocytopenia, hypoparathyroidism or hyperphosphatemia or may be used to increase stem cell mobilization in a subject. Any mode of administration (e.g., oral, intravenous, intramuscular, ophthalmic, topical, dermal, subcutaneous, and rectal) can be used in the treatment methods of the invention. A physician will determine appropriate dosing for the patient being treated, which will depend in part on the size of the patient, the severity of the disease or condition, and the particular disease or condition being treated.

Formulation of pharmaceutical compositions

The administration of any compound described herein (e.g., PTH-derived peptides) or identified using the methods of the invention may be by any suitable means that results in a concentration of the compound that treats the subject disease condition. The compound may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is suitable for the oral, parenteral (e.g., intravenously or intramuscularly), rectal, cutaneous, nasal, vaginal, inhalant, skin (patch), ocular, or intracranial administration route. Thus, the composition may be in the form of, e.g., tablets, ampules, capsules, pills, powders, granulates, suspensions, emulsions, solutions, gels including hydrogels, pastes, ointments, creams, plasters, drenches, osmotic delivery devices, suppositories, enemas, injectables, implants, sprays, or aerosols. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: *The Science and Practice of Pharmacy*, 20th edition, 2000, ed. A.R. Gennaro, Lippincott Williams & Wilkins, Philadelphia, and *Encyclopedia of Pharmaceutical Technology*, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Pharmaceutical compositions may be formulated to release the active compound immediately upon administration or at any predetermined time or

time period after administration. The latter types of compositions are generally known as controlled release formulations, which include (i) formulations that create substantially constant concentrations of the agent(s) of the invention within the body over an extended period of time; (ii) formulations that after a predetermined lag time create substantially constant concentrations of the agents of the invention within the body over an extended period of time; (iii) formulations that sustain the agent(s) action during a predetermined time period by maintaining a relatively constant, effective level of the agent(s) in the body with concomitant minimization of undesirable side effects associated with fluctuations in the plasma level of the agent(s) (sawtooth kinetic pattern); (iv) formulations that localize action of agent(s), e.g., spatial placement of a controlled release composition adjacent to or in the diseased tissue or organ; (v) formulations that achieve convenience of dosing, e.g., administering the composition once per week or once every two weeks; and (vi) formulations that target the action of the agent(s) by using carriers or chemical derivatives to deliver the compound to a particular target cell type. Administration of the compound in the form of a controlled release formulation is especially preferred for compounds having a narrow absorption window in the gastro-intestinal tract or a relatively short biological half-life.

Any of a number of strategies can be pursued in order to obtain controlled release in which the rate of release outweighs the rate of metabolism of the compound in question. In one example, controlled release is obtained by appropriate selection of various formulation parameters and ingredients, including, e.g., various types of controlled release compositions and coatings. Thus, the compound is formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the compound in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, molecular complexes, microspheres, nanoparticles, patches, and liposomes.

Parenteral Compositions

The composition containing compounds described herein or identified using the methods of the invention may be administered parenterally by injection, infusion, or implantation (subcutaneous, intravenous, intramuscular, intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation.

Compositions for parenteral use may be provided in unit dosage forms (e.g., in single-dose ampoules), or in vials containing several doses and in which a suitable preservative may be added (see below). The composition may be in form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the active agent(s), the composition may include suitable parenterally acceptable carriers and/or excipients. The active agent(s) may be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing agents.

As indicated above, the pharmaceutical compositions according to the invention may be in a form suitable for sterile injection. To prepare such a composition, the suitable active agent(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, dextrose solution, and isotonic sodium chloride solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl, or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in

water, a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol or the like.

The following examples are intended to illustrate rather than limit the invention.

Example 1

Identification of short-lived and long-acting PTH peptides

Characterization of ligands using a competitive binding assay. To identify PTHR ligands, kinetic dissociation experiments were first performed to examine the stability of complexes formed between PTH and PTHrP radioligand analogs and the human PTHR expressed in membranes prepared from HKRK-B7 cells. For each radioligand, dissociation was examined in the presence and absence of GTPγS, so as to assess the effects of functionally uncoupling the receptor from heterotrimeric G proteins (Figures 1A-1C). For ^{125}I-PTH(1-34) and ^{125}I-PTHrP(1-36) (Figures 1A and 1B, respectively), the dissociation data, both in the absence and presence of GTPγS (solid and open symbols, respectively), were better fit by a two-phase decay equation than by a single-phase equation. For ^{125}I-PTH(1-34) and in the absence of GTPγS, 17% of the complexes were unstable and decayed rapidly ($t_{1/2}$<1 min), whereas the remaining 83% were stable and decayed slowly ($t_{1/2}$ ~ 4 h). Upon the addition of GTPγS, the rapid, unstable component increased to 21%, such that 77% of the complexes remained stable ($t_{1/2}$ ~ 2 h) (Figure 1A). These findings with ^{125}I-PTH(1-34) agree closely with previous dissociation studies performed on this radioligand, and highlight the capacity of PTH(1-34) to bind to a high affinity, G protein-uncoupled PTHR conformation (R^0) (Shimizu et al., *J. Biol. Chem.* 280:1797-807 (2005); Dean et al., *Mol. Endocrinol.* 20:931-43 (2006)). The complexes formed with ^{125}I-PTHrP(1-36) and the PTHR were again mostly stable in the absence of GTPγS (68% decayed with a $t_{1/2}$ of ~3 h). By contrast, most of the complexes became unstable upon addition of GTPγS (72% decayed with a $t_{1/2}$ of ~1 minute; Figure 1B). This rapid dissociation of

^{125}I-PTHrP(1-36) from the PTHR induced by GTPγS addition mirrors that observed previously for ^{125}I-[Aib^{1,3},M]PTH(1-15) (Dean et al., *Mol. Endocrinol.* 20:931-43 (2006)); each of these radioligands thus appears to bind predominantly to the PTHR in a G protein-coupled conformation (RG).

The structural differences in PTH(1-34) and PTHrP(1-36) that underlie the functional differences seen for the two ligands in the above dissociation studies then identified. The divergent residues at position 5 in PTH and PTHrP (Ile and His, respectively) have been shown to play important roles in determining the affinity (Shimizu et al., *J. Biol. Chem.* 280:1797-807 (2005); Gardella et al., *J. Biol. Chem.* 270:6584-6588 (1995)) and subtype selectivity (Gardella et al., *J. Biol. Chem.* 271:19888-19893 (1996); Behar et al., *Endocrinology* 137:4217-4224 (1996)) with which these ligands bind to the receptor. The receptor-dissociation properties of ^{125}I-Ile^5-PTHrP(1-36) were examined, again in the absence and presence of GTPγS. This radioligand dissociated from the receptor slowly, both in the presence and absence of GTPγS, and, in each case, with mono-phasic kinetics ($t_{1/2} > 2$ h; Figure 1C). Thus, the His^5 Ile substitution markedly enhanced the stability with which PTHrP binds to the PTHR, in the G protein-coupled, and especially in the G protein-uncoupled state.

Effects of GTPγS on equilibrium binding. The effects of GTPγS on the binding of these radioligands to the PTHR under approximate-equilibrium conditions was assessed. by incubating with cell membranes for 90 minutes in the absence or presence of GTPγS at varying concentrations. The binding of ^{125}I-PTH(1-34) and ^{125}I-Ile^5-PTHrP(1-36) to membranes prepared from HKRK-B7 cells was largely unaffected by GTPγS (< ~20% inhibition at 1 x 10^{-4} M GTPγS), whereas the binding of ^{125}I-PTHrP(1-36) was strongly inhibited by GTPγS (~70% inhibition at 1 x 10^{-7} M GTPγS; IC_{50} = 1 x 10^{-9} M; Figure 2A). To assess binding to the rat PTHR, parallel studies were performed using membranes prepared from the rat osteoblastic cell line ROS17/2.8, which endogenously expresses the rat PTHR. As with the human PTHR in HKRK-B7 cell membranes, the binding of ^{125}I-Ile^5-PTHrP(1-36) to rat PTHR likewise

was largely insensitive to GTPγS (Figure 2B). The binding of ^{125}I-PTH(1-34) to the rat PTHR appeared more sensitive to GTPγS than was its binding to the human PTHR (Figure 2A vs. 2B), although the majority of the binding was resistant to the nucleotide analog. As for the human PTHR, GTPγS strongly inhibited the binding of ^{125}I-PTHrP(1-36) to the rat PTHR, which was as sensitive to the nucleotide analog as the binding of ^{125}I-[Aib^{1,3},M]PTH(1-15) (Figure 2B). Thus, PTH(1-34) and Ile^5-PTHrP(1-36) bind more strongly to the G protein-uncoupled conformation of the PTHR (R^0) than does PTHrP(1-36) or [Aib^{1,3},M]PTH(1-15). By contrast, the later two peptides bind preferentially to the G protein-coupled conformation, RG.

Competition methods were then used to analyze the relative affinities with which PTH and PTHrP ligands bind to the RG and R^0 receptor conformations of the PTHR. To assess binding to RG, ^{125}I-[Aib^{1,3},M]PTH(1-15) was used as a tracer radioligand, as this peptide binds predominantly to RG. Membranes were prepared from COS-7 cells co-transfected with the hPTHR and a negative-dominant $G\alpha_s$ subunit ($G\alpha_s$ND), which enriches for RG, related to R and R^0, as described previously (Dean et al., *Mol. Endocrinol.* 20:931-943 (2006); Berlot, C.H., *J. Biol. Chem.*277:21080-21085 (2002); Dean et al., *J. Biol. Chem.* 281:32485-32495(2006)). To assess binding to R^0, ^{125}I-PTH(1-34) was used as a radioligand (binds predominantly to R^0). Membranes were prepared from COS-7 cells transfected with the hPTHR alone. GTPγS (1×10^{-5}) was added to the binding reactions so as to functionally uncouple receptor-heterotrimeric G protein complexes, thus enriching for the R^0 (and R) conformations, relative to RG. The relative apparent affinities obtained for several unlabeled PTH and PTHrP ligand were then compared in these two assays, to assess the selectivity with which each of the ligands bound to the R^0 vs. RG PTHR conformation.

PTH(1-34) bound to the R^0 conformation with a five-fold weaker affinity than it did to the RG conformation (IC_{50} = 4.2 nM vs. 0.86 nM, P = 0.0002; Figure 3A, Table 5). PTHrP(1-36) exhibited greater selectivity as it bound to R^0 with a 66-fold weaker affinity than it did to RG (P = 0.04; Figure

3B; Table 5). Thus its selectivity for RG (vs. R^0) was 13-fold greater than that of PTH(1-34). Reciprocal exchange of residue 5 in the ligands reversed this pattern of conformational selectivity; thus, His^5-PTH(1-34) bound to R^0 with a 750-fold weaker affinity than it did to RG, and Ile^5-PTHrP(1-36) bound to R^0 with only a three-fold weaker affinity than it did to RG ($P < 0.002$; Figs. 3C and 3D; Table 5).

Table 5. Competition binding to the RG and R^0 conformations of the human PTH receptor

	IC_{50} (nM)				
	RG		R^0		
	^{125}I-PTH(1-15) +G_SND		^{125}I-PTH(1-34) +GTPγS		*R0:RG*
		n		*n*	
[$Nle^{8,21}$,Tyr^{34}]rPTH(1-34)NH_2	0.86 ± 0.24	7	4.2 ± 0.5	7	5
[His^5,$Nle^{8,21}$,Tyr^{34}]rPTH(1-34)NH_2	0.094 ± 0.019	4	71 ± 7	4	753
[Tyr^{36}]hPTHrP(1-36)NH_2	0.42 ± 0.09	3	28 ± 6	3	66
[Ile^5,Tyr^{36}]hPTHrP(1-36)NH_2	0.92 ± 0.07	3	2.9 ± 0.1	3	3
rPTH(1-34)NH_2	0.34 ± 0.16	3	2.3 ± 0.3	3	7
[His^5]rPTH(1-34)NH_2	0.19 ± 0.04	5	26 ± 5	5	138
hPTH(1-34)NH_2	0.39 ± 0.24	3	6.6 ± 2.4	3	17
[His^5]hPTH(1-34)NH_2	0.76 ± 0.04	5	122 ± 35	5	160
hPTHrP(1-36)NH_2	0.59 ± 0.02	3	24 ± 3	3	42
[$Aib^{1,3}$,M]rPTH(1-15)NH_2	0.74 ± 0.18	3	1029 ± 148	3	1,397

The $Ile^5 \rightarrow$His substitution also strongly reduced affinity for R^0 without greatly affecting affinity for RG in human-PTH(1-34) and rat-PTH(1-34) peptides that lacked the methionine$^{8,21} \rightarrow$norleucine and Phe^{34} Tyr^{34} substitutions of our control PTH(1-34) analog (Figures 6A, 6B, 6D, and 6E and Table 4). Thus, PTH(1-34) binds with higher affinity to R^0 than does PTHrP(1-36), whereas both PTH(1-34) and PTHrP(1-36) bind with high affinity to the RG PTHR conformation. Residue 5 in the ligand plays a significant role in modulating the capacity of the ligands to bind to the R^0 versus RG conformations. In addition, residues carboxy-terminal of position 15 in PTH(1-34) contribute to the capacity of the ligand to bind strongly to R^0,

as shown by [Aib^{1,3},M]PTH(1-15), which binds only weakly to R^0 but maintains strong affinity for RG (Figure 6C and Table 4).

Direct recording of PTHR activation. The fluorescent resonance energy transfer (FRET) approach has recently been used to assess, in real time and in intact cells, the processes of ligand binding and receptor activation for the PTHR. This approach was therefore used as an independent means to compare the time courses by which PTH and PTHrP ligands interact with the PTHR. The approach used exploits an intramolecular FRET signal that occurs in a human PTHR construct, PTHR-CFP_{IC3}/YFP_{CT} (formerly called PTHR-cam). This construct contains cyan-fluorescent protein (CFP) in the third intracellular loop and yellow-fluorescent protein (YFP) in the carboxy-terminal tail. A FRET signal is produced by PTHR-CFP_{IC3}/YFP_{CT} in the basal state, and this signal diminishes upon agonist binding, likely due to conformational change that occurs upon activation.

hPTH(1-34) induced a rapid ($t_{1/2}$ = 0.7 sec) reduction (~13%) in the FRET signal produced by cells expressing PTHR-CFP_{IC3}/YFP_{CT} (Figure 4A). The FRET signal remained suppressed during the 15 seconds of ligand application, as well as for at least 60 seconds after the ligand-containing buffer was exchanged for a ligand-free buffer (ligand application times are marked by the black horizontal line above the graphs in Figures 4A-4C). The FRET response profile obtained for hPTH(1-34) replicates the profile observed for this ligand in previous FRET studies (Vilardaga et al., *Nat. Biotechnol.* 21:807-812 (2003)). The amino-terminal peptide, [Aib^{1,3},M]PTH(1-14), induced a FRET response with slightly faster kinetics ($t_{1/2}$ = 0.5 sec) and with a shallower magnitude (~5 %) than that produced by hPTH(1-34) (Figure 4B). Moreover, the FRET response produced by [Aib^{1,3},M]PTH(1-15) began to decay immediately upon exchange of the buffer to a ligand-free one (Figure 4B). PTHrP(1-36) induced a relatively slow FRET response ($t_{1/2}$ = ~2 to 5 seconds), and the signal began to decay immediately upon changing to a ligand-free buffer (Figure 4C). The Ile^5-substituted ligand Ile^5-PTHrP(1-36) induced a FRET signal that was remarkably similar to that of PTH(1-34), in that the

response was rapid ($t_{1/2}$ = 0.5 - 0.7 sec), and stable after ligand removal (Figure 4D). These kinetic data, derived by a spectroscopic approach, fully agree with those obtained in the above binding radioligand dissociation assays, thus indicating that PTH(1-34) and PTHrP(1-36) bind predominantly to distinct conformations of the PTHR. They also confirm the important role of residue five in the ligands in contributing to this conformational selectivity.

cAMP measurements in HKRK-B7 cells. Given that LR^0 complexes can isomerize to LRG complexes, a potential consequence of stable binding of a ligand to R^0 is a prolongation of the signaling response induced by that ligand, relative to a ligand that only poorly stabilizes R^0. To examine this possibility, the capacity of PTH and PTHrP ligands to produce sustained cAMP responses in PTHR-expressing cells was assessed. Cells were thus treated with a ligand for ten minutes, washed to remove unbound ligand. At various times after washing, IBMX was applied for five minutes, and the resulting intracellular cAMP was measured. Using this approach, only the cAMP produced during the final, five minute IBMX incubation phase is measurable. The experiments of Figure 5A compare the time courses of the cAMP responses produced by PTHrP(1-36) and Ile^5-PTHrP(1-36) in HKRK-B7 cells. Immediately after the wash-out step, cells treated with either ligand produced approximately the same amount of cAMP, which was ~100-fold above the basal cAMP level in untreated cells. Two hours after the wash-out step, the cells treated with Ile^5-PTHrP(1-36) maintained a cAMP signaling capacity that was ~50% of the signaling capacity seen immediately after ligand wash-out (Figure 5A). By contrast, the signaling capacity of cells treated with PTHrP(1-36) at two hours was ~19% of the initial response, and thus ~65% less than the response observed at two hours for Ile^5-PTHrP(1-36) ($P \leq 0.003$). PTH(1-34) produced responses at each time point that were nearly identical to those produced by Ile^5-PTHrP(1-36) ($P => 0.05$, data not shown). Thus, the cAMP signaling responses induced by PTH(1-34) and Ile^5-PTHrP(1-36) decayed about twice as slowly as did that of and PTHrP(1-36) ($t_{1/2}$ = ~ 2 h vs. ~1 h). These differences in the duration of the cAMP signaling capacity observed for the PTH and

PTHrP analogs parallel the differences seen in the rates with which the corresponding radioligands dissociated from the PTHR in the presence of GTPγS (Figures 1A-1C).

cAMP measurements in HKRK-B64 cells. The capacity of the ligands to produce sustained (or delayed) cAMP signaling responses was further examined in HKRK-B64 cells, which express the hPTHR at a more physiological level than do HKRK-B7 cells (90,000 per cell vs. 950,000/cell). Time course experiments indicated that differences in the duration of ligand-induced signaling responses were best resolved in these cells 60 minutes after ligand wash-out (data not shown). In these experiments, a maximum response was determined for each peptide by incubating the cells concomitantly with ligand and IBMX for 10 minutes (no wash-out phase); the cAMP responses observed at 60 minutes after ligand washout were then expressed as a percentile of the corresponding maximum response.

As in HKRK-B7 cells, PTH(1-34) and Ile^5-PTHrP(1-36) produced cAMP responses at 60 minutes after wash-out that were 47% and 40% of their corresponding maximum responses, respectively, in HKRK-B64 cells (Figure 5B). The analogs His^5-PTH(1-34) and PTHrP(1-36) produced responses at 60 minutes that were 34% and 19% of their maximum response. The response induced by [$Aib^{1,3}$,M]PTH(1-15) at two hours was 23% of its maximum response, and thus was comparable to that of PTHrP(1-36) (P = 0.7). Different PTH and PTHrP ligand analogs that exhibit the same or comparable activities when assessed in acute dose-response signaling assays (Figure 7; Table 6), can produce quantitatively different cumulative signaling responses in cells, that are most likely due to the capacity of the ligands to form a stable complex with the receptor.

Table 6. cAMP and IP signaling properties of PTH and PTHrP ligands.

	cAMP in HKRK-B64 cells [a]		*IP in COS-7/hPTHR cells* [c]	
	EC_{50} (nM)	E_{max} [b] (picomole/well)	EC_{50} (nM)	E_{max} [d] (cpm/well)
[Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2	5.1 ± 0.5	55 ± 12	18 ± 3	2,407 ± 138
[His^{5},Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2	2.7 ± 0.6 [e]	59 ± 12	30 ± 12	2,231 ± 229
[Tyr^{36}]hPTHrP(1-36)NH_2	5.6 ± 1.3	62 ± 15	23 ± 8	2,514 ± 270
[Ile^{5},Tyr^{36}]hPTHrP(1-36)NH_2	5.4 ± 1.9	61 ± 14	23 ± 7	2,793 ± 303

a data are means (±s.e.m.) from four experiments; *b* basal camp (not subtracted) was 5.2 ± 0.9 pmole/well).
c data are means (±s.e.m.) from five experiments; *d* basal IP value (not subtracted) was 330±8 cpm/well.
e, *P* vs. [Nle^{8,21},Tyr^{34}]rPTH(1-34)NH_2 = 0.02.

cAMP measurements in rat osteoblastic cells. The capacity of certain ligands to produce cAMP signaling responses was further examined in vitro using rat osteoblastic cells (ROS17/2.8 cell line; Figure 8). ROS 17/2.8 cells were treated with hPTH(1-28)NH_2; Ala^{1,12},Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2; hPTH(1-34)NH_2, or r(rat)PTH(1-34)NH_2 for 10 minutes at room temperature in the presence of IBMX, and the resulting intracellular cAMP formed was quantified by radioimmuno assay. The EC_{50} values for the various peptides were 7.39 nM for hPTH(1-28)NH_2; 0.37 nM for Ala^{1,12},Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH2; 0.31 nM for hPTH(1-34)NH_2; and 0.021 nM for r(rat)PTH(1-34)NH_2.

cAMP plasma measurements in mice in vivo. Wild-type mice were injected subcutaneously with vehicle (0.9% NaCl/0.05% Tween-20), or vehicle containing a PTH peptide so as to achieve a concentration ranging from 10 to 1000 nmol/kg of body weight. At the indicated times after injection, blood was withdrawn from the tail vein, and the amount of cAMP in the resulting plasma was quantified by radioimmuno assay (Figures 9A-9D).

The mice were further analyzed for changes in plasma phosphate and serum ionized calcium concentrations. Wild-type mice were injected subcutaneously with vehicle (0.9% NaCl/0.05% Tween-20), or vehicle containing Ala^{1,12},Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2 or hPTH(1-34)NH_2 at doses of 50 nmol/kg body weight. At the indicated times after injection, blood was withdrawn from the tail vein and the concentrations of plasma phosphate (Figure 10A) and serum ionized calcium (Figure 10B) were

determined. Serum ionized calcium concentrations were determined using a Chiron Diagnostics Model 634 Ca^{++}/pH analyzer. Plasma phosphate concentrations were measured using a Phosphorous Liqui-UV assay kit (StanBio Laboratory, Boerne, TX). Both peptides resulted in similar maximal increases in serum calcium and similar maximal reductions in plasma phosphate, but that the responses to $Ala^{1,12}$,Aib^{3},Gln^{10},Har^{11},Trp^{14},Arg^{19}-hPTH(1-28)NH_2 were more prolonged than those to hPTH(1-34)NH_2.

Phosphate uptake inhibition in opossum kidney cells. Inhibition of phosphate uptake was assessed using the opossum kidney (OK) cell line, which are derived from the renal proximal tubule. These cells mediate sodium-dependent phosphate transport function which is regulated by PTH receptor ligands. Thus, treating OK cells with PTH(1-34) inhibits their uptake of phosphate from the culture media.

Brief (10 minute) exposure of the cells to A^{1},Aib^{3},M-PTH(1-28) results in a dramatically prolonged inhibitory effect on phosphate uptake, whereas PTH(1-34) and hPTHrP(1-36) peptides exhibit a much shorter duration of phosphate uptake inhibition (Figure 11).

Pharmacokinetics and hypercalcemic action of PTHR ligands in normal rats. Pharmacokinetic profiles of iv injected PTHrP(1-36) and [I^{5}]-PTHrP(1-36) were investigated in normal rats (Figure 12). Both PTHrP(1-36) and [I^{5}]-PTHrP(1-36) rapidly disappeared from the circulation, and the pharmacokinetic profile of [I^{5}]-PTHrP(1-36) was comparable to that of PTHrP(1-36).

We also measured the calcemic actions of intravenously injected PTHrP(1-36) and [I^{5}]-PTHrP(1-36) in normal rats (Figure 13). PTHrP(1-36) and [I^{5}]-PTHrP(1-36) at 20 and 80 nmol/kg increased blood ionized calcium levels to the same extent at one hour. Blood ionized calcium levels declined at two hours after injection with PTHrP(1-36), but were sustained at high levels at two hours after injection with [I^{5}]-PTHrP(1-36). Thus, [I^{5}]-PTHrP(1-36) and PTHrP(1-36) exhibited comparable pharmacokinetic profiles (Figure 12), but [I^{5}]-PTHrP(1-36) exhibited a higher binding affinity for the R^{0} PTHR conformation *(Figs. 3 and 6)*. Therefore, the prolonged calcemic actions of

[I^5]-PTHrP(1-36) observed in vivo can best be explained by its high R^0 binding affinity.

In vitro and in vivo screening of PTH or PTHrP analogs with human PTH receptor. We designed and synthesized native PTH-PTHrP hybrid analogs, and [$A^{1,3,12}$, Q^{10},R^{11},W^{14}] (M-modified) PTH-PTHrP hybrid analogs, and tested their cAMP signaling capacities in HKRK-B7 cells expressing the hPTH receptor. Each of the native, and M-modified PTH/PTHrP hybrid analogs showed cAMP signaling activity comparable to hPTH(1-34) (Figure 25). We assessed affinity of native or M-modified PTH and PTHrP hybrid analogs for the R^0 and RG states of the human PTH receptor (Figures 26A and 26B) in COS-7 cell membranes.

Hypercalcemic action of PTH and PTHrP analogs in normal and TPTX rats. The transient calcemic actions of the native and M-modified PTH-PTHrP hybrid analogs were evaluated in normal and TPTX rats using PTH(1-34) and PTHrP(1-36) as controls (Figures 13A, 14A, 15A, 15B, 16A, 17A, and 18A). I^5-PTHrP(1-36), MPTH(1-14)/PTHrP(15-36), PTH(1-14)/PTHrP(15-36), PTH(1-18)/PTHrP(19-36), M-PTH(1-34) showed higher calcemic actions than did PTH(1-34); in contrast, PTH(1-22)/PTHrP(23-36) and PTH(1-26)/PTHrP(27-36) showed weaker calcemic actions than did either PTH(1-34) or PTHrP(1-36) control peptides. Binding to the rat PTHR was also measured in vitro. Length of signaling activity was confirmed using the delayed cAMP assay (Figures 13B-13C, 14B-14C, 15B, 16B-16C, 17B-17C, and 18B), which clearly demonstrates a correlation between the R^0/RG selectivity from binding data shown in vitro and both the hypercalcemic action in vivo as well as and delayed cAMP response in vitro. The cAMP signaling of all these peptides did not vary substantially (Figures 19A, 19B, 20A, and 20B).

Materials and methods

The following materials and methods were used to perform the above experiments.

Peptides. The peptides used in Figures 1-3, and 5-11 were synthesized by the M.G.H. Biopolymer Core facility, as described in Shimizu et al., *J. Biol. Chem.* 276:49003-49012 (2001). These peptides include $[Nle^{8,21},Tyr^{34}]$rat(r)PTH(1-34)NH_2 (PTH(1-34); $[Aib^{1,3},Nle^{8},Gln^{10},homoarginine^{11},Ala^{12},Trp^{14},Tyr^{15}]$rPTH(1-15)$NH_2$ ($[Aib^{1,3},M]$PTH(1-15); $[Ala^{1,12},Aib^{3},Gln^{10},homoarginine^{11},Trp^{14},Arg^{19}]$human(h)PTH(1-28)$NH_2$ {$[Ala^{1},Aib^{3},M]$PTH(1-28)}; $[Tyr^{36}]$hPTHrP(1-36)NH_2 {(PTHrP(1-36)}; $[Ile^{5},Tyr^{36}]$hPTHrP(1-36)NH_2 {Ile^{5}-PTHrP(1-36)}; hPTH(1-34)NH_2; $[His^{5}]$hPTH(1-34)NH_2; rPTH(1-34)NH_2 and $[His^{5}]$rPTHrP(1-36)NH_2. The hPTH(1-34)COOH peptide (free carboxyl) used in FRET analyses (Figure 4) was purchased from Bachem California (Torrance, CA). The rat studies used human PTHrP(1-36) synthesized by American Peptide Company, Inc. (California, USA). Human PTH(1-34) was purchased from Peptide Institute Inc (Osaka, Japan). PTH or PTHrP analogs were synthesized by Sigma Aldrich Japan (Tokyo, Japan). Peptides used in rat studies were dissolved at 1 mM in 10 mM acetic acid, and stocked at -80 °C refrigerator.

The peptides used in Figures 12-16 were purchased from either American Peptide Company, Inc., California, USA (hPTHrP(1-36)COOH), Peptide Institute Inc., Osaka, Japan (hPTH(1-34)COOH), or Sigma-Aldrich Japan, Tokyo, Japan (PTH/PTHrP hybrid analogs). All peptides were dissolved in 10 mM acetic acid to a peptide concentration of between 0.1 mM and 4 mM; and stored at -80°C. Peptide purity and quality was verified by analytical high performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Radiolabeled peptide variants were prepared by the oxidative chloramine-T procedure using $Na^{125}I$ (specific activity: 2,200 Ci/mmol, Perkin Elmer/NEN Life Science Products, Boston, MA) and were purified by reversed-phase HPLC.

Cell Culture. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (HyClone, Logan UT), 100

units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen Corp. Carlsbad, CA). The PTHR-expressing cell lines used were HKRK-B7, HKRK-B64, ROS 17/2.8, and HEK-PTHR-cam. The HKRK-B7 and HKRK-B64 lines were derived from the porcine kidney cell line, LLC-PK1, via stable transfection with plasmid DNA (pCDNA1 vector, Invitrogen Corp.) encoding the human PTHR, and express the PTHR at approximate surface densities of 950,000 and 90,000 PTH-binding sites per cell, respectively (Takasu et al., *J. Bone Miner. Res.* 14:11-20 (1999)). ROS 17/2.8 cells are rat osteosarcoma cells (Majeska et al., *Endocrinology* 107:1494-1503 (1980)) and express the endogenous rat PTHR at an approximate surface density of 70,000 PTH-binding sites per cell (Yamamoto, I. et al., *Endocrinology* 122:1208-1217 (1988)). HEK-PTHR-cam cells were derived from HEK-293 cells by stable DNA transfection and express a human PTHR derivative (PTHR-cam) containing cyan fluorescent protein (CFP) inserted at Gly^{395} in the third intracellular loop and yellow fluorescent protein (YFP) inserted in the carboxy-terminal tail (Vilardaga et al., *Nat. Biotechnol.* 21:807-812 (2003)). Cells were propagated in T75 flasks and divided into 24-well plates for assays with intact cells, six-well plates for membrane preparations, or onto glass cover-slips for FRET studies. COS-7 cells were transiently transfected in six-well plates using Fugene-6 (Roche Diagnostics, Indianapolis IN) and CsCl-purified plasmid DNA encoding the PTHR (3 μl Fugene, 1 μg DNA, per well), or co-transfected with plasmids encoding the PTHR and a negative-dominant $G\alpha_s$ subunit $G\alpha_sND$ (6 μl Fugene, 1 μg each DNA per well). This $G\alpha_sND$ subunit binds more effectively, but unproductively, to receptors than does wild-type $G\alpha_s$ (Berlot, C.H. *J. Biol. Chem.* 277:21080-21085 (2002)), and has been found to enhance the binding of ^{125}I-[$Aib^{1,3}$,M]PTH(1-15)NH_2 radioligand to the PTHR in cell membranes (see below) (Dean, T. et al., J. Biol. Chem. (2006)).

Binding Studies. Binding studies were performed using cell membranes as described (Dean et al., *Mol Endocrinol* 20(4):931-43 (2006)). Briefly, reactions were incubated at room temperature in membrane assay buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl, 3 mM $MgSO_4$, 20% glycerol, 3 mg/ml

bovine serum albumin, protease inhibitor cocktail --final concentrations: 1 mM AEBSF, 0.8 μM Aprotonin, 20 μM leupeptin, 40 μM Bestatin, 15 μM Pepstatin A, 14 μM E-64-- Sigma-Aldrich Inc., St. Louis, MO). Reactions contained a total membrane protein concentration of 20 to 100 μg/mL, and a total radioactivity concentration of approximately 150,000 cpm/ml. Unlabeled peptide ligands and/or GTPγS (Sigma-Aldrich Inc. St. Louis, MO) were added to the reactions as indicated. At the end of the reaction, bound and free radioligand were separated by vacuum filtration using a 96-well vacuum filter plate and vacuum filter apparatus (Multi-Screen system with Durapore HV, 0.65 μM filters; Millipore Corp., Milford, MA); the air-dried filters were then detached from the plate and counted for gamma radioactivity using a gamma counter.

Radioligand dissociation. These studies were performed as bulk reactions in 15 mL round-bottom polystyrene snap-cap tubes (Falcon) (total reaction volume = 5.0 ml). Membranes and radioligand were pre-incubated for 90 minutes to allow complex formation; the dissociation phase was then initiated by the addition of an excess of the unlabeled analog of the radioligand (5×10^{-7} M final concentration), with or without GTPγS (5×10^{-5} M). Immediately prior to this addition (t=0), and at successive time-points thereafter, 0.2 ml aliquots (~30,000 cpm) were withdrawn and immediately processed by vacuum filtration, as described above. Non-specific binding was determined in parallel reaction tubes containing the unlabeled analog of the radioligand (5×10^{-7} M) in both the pre-incubation and dissociation phases. The specifically bound radioactivity at each time point was calculated as a percent of the radioactivity specifically bound at t = 0.

Equilibrium competition binding and GTPγS inhibition. Binding reactions performed with ^{125}I-[Aib^{1,3},M]PTH(1-15) radioligand were assembled and incubated in the wells of the 96-well, Multi-Screen vacuum filtration plates. Membranes, tracer radioligand, and various concentrations of unlabeled ligands and/or GTPγS were incubated in the wells for 90 minutes, following which, the reaction plates were processed by rapid vacuum filtration to separate

bound from free radioligand, as described above. Binding reactions performed with ^{125}I-PTH(1-34) radioligand were assembled and incubated in 96-well polystyrene micro-titer plates (Falcon, total reaction volume = 230 µl), and at the end of the incubation were transferred to wells of a 96-well, Multi-Screen vacuum filtration plate and processed, as described above. This transfer maneuver was performed for the ^{125}I-PTH(1-34)-containing reactions to minimize non-specific binding of the radioligand to the Multi-screen filter membranes. For both radioligands, the non-specific binding was determined in reactions containing a saturating concentration of the unlabeled analog of the radioligand. The specifically bound radioactivity was calculated as a percent of the radioactivity specifically bound in the absence of a competing ligand or GTPγS.

To assess the capacities of various unlabeled peptide ligands to bind to the G protein-uncoupled and G protein-coupled PTHR conformations (R^0 and RG, respectively), membranes were prepared from transiently transfected COS-7 cells and the following assay conditions. To assess binding to R^0, membranes were prepared from cells transfected with the PTHR, ^{125}I-PTH(1-34) as a tracer radioligand, and GTPγS (1×10^{-5} M) was added to the binding reactions. This binding format is based on the premise that ^{125}I-PTH(1-34) binds predominantly to the R^0 conformation of the PTHR, and that this conformation is enriched in the membranes, relative to RG, by the presence of GTPγS (Hoare et al., *J. Biol. Chem.* 276:7741-53 (2001); Dean et al., *Mol Endocrinol* (2006)). To assess binding to RG, membranes prepared from cells co-transfected with the PTHR and a negative dominant $G\alpha_S$ subunit ($G\alpha_S$ND) were used, and ^{125}I-[Aib^{1,3},M]PTH(1-15) was used as a tracer radioligand. This binding format is based on the premise that ^{125}I-[Aib^{1,3},M]PTH(1-15) binds predominantly to the RG conformation of the PTHR, and that this conformation is enriched in the membranes, relative to R or R^0, by the presence of $G\alpha_S^{ND}$ (Hoare, S. *J. Biol. Chem.* (2001); Berlot, C.H. *J. Biol. Chem.* (2002); Dean, T. et al., *J Biol. Chem.* (2006)). Analysis of binding to any low affinity PTHR conformation (R)

present in the membrane preparations is precluded by the low concentrations (~25 pM) of tracer radioligands in the reactions.

Fluorescent Resonance Energy Transfer (FRET). HEK-293 cells stably expressing HEK-PTHR-CFP_{IC3}/YFP_{CT} (previously called HEK-PTHR-Cam cells (Vilardaga et al., *Nat. Biotechnol.* 21:807-812 (2003)) were grown on glass coverslips and processed for FRET analysis as described. With these cells, excitation of the CFP (donor) in PTHR-CFP_{IC3}/YFP_{CT} with ultraviolet light ($\lambda_{max.ex.}$ = 436 nm; $\lambda_{max.em.}$ = 480 nm) produces an intramolecular FRET to the YFP (acceptor), resulting in emission from that YFP ($\lambda_{max.ex.}$ = 480 nm, $\lambda_{max.em.}$ = 535 nm). This FRET response is observable as a decrease in intensity of CFP light emission at 480 nm, and an increase in intensity of YFP light emission at 535 nm. The FRET signal is produced by PTHR-CFP_{IC3}/YFP_{CT} in the ground-state receptor and decreases upon binding of an agonist. PTH ligands were added to the cells, and washed from the cells using a computer-assisted, solenoid valve-controlled, rapid superfusion device (ALA Scientific Instruments, Westbury, NY); solution-exchange times were 5 ms to 10 ms. Fluorescence was monitored using a Zeiss inverted microscope equipped with a 100x objective and a dual emission photometric system (Til Photonics), coupled to an avalanche photodiode detection system and an analog-digital converter (Axon Instruments). The FRET signal detected upon excitation at 436 nm was calculated as the normalized FRET ratio: $F_{YFP(535\ nm)}/F_{CFP(480\ nm)}$ where $F_{YFP(535\ nm)}$ is the emission at 535 nm, corrected for spillover of the CFP signal into the YFP channel, and $F_{CFP(480nm)}$ is the emission at 480 nm, corrected for spillover (minimal) of the YFP emission into the CFP channel. Changes in fluorescence emissions due to photo-bleaching were subtracted.

Stimulation of Intracellular cAMP. Following treatment of cells with a ligand, the intracellular cAMP levels were measured by radioimmuno assay, as described (Shimizu et al., *J. Biol. Chem.* 276:49003-49012 (2001)). The capacities of ligands to produce a delayed cAMP response in cells after a brief exposure to the ligand was assessed as follows. The cells in 24-well plates were rinsed in binding buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM

KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum) and then incubated in binding buffer with or without a peptide ligand (1×10^{-7} or 3×10^{-7} M) for 10 minutes at room temperature; the buffer was then removed, the cells were washed three times with binding buffer, incubated further in binding buffer for varying times (1 to 120 minutes); the buffer was then replaced by binding buffer containing IBMX (2 mM), and after an additional five minute incubation, the intracellular cAMP was quantified. By this approach, which has been used previously for the PTH receptor (Tawfeek, H., and Abou-Samra, A., *J. Bone Miner. Res.* 14:SU444 (1999); Biselo et al., *J. Biol. Chem.* 277:38524-38530 (2002)), only the cAMP produced during the final IBMX-containing stage of the incubation is measurable, because cAMP produced prior to IBMX addition is degraded by cellular phosphodiesterases.

In the cAMP experiments of Figure 14, HKRK-B7 were seeded in 96 well plates at 1×10^5 cells/well and incubated overnight. On the following day, the cells were washed once with 200 µl of binding buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum), followed by addition of 100 µl cAMP assay buffer (DMEM, 2 mM IBMX, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) on ice. Then, 50 µl of binding buffer containing varying amounts of human PTH(1-34), human PTHrP(1-36), or PTH analogs (final volume = 150 µl), were added to each well, and placed in a water bath at 37°C, and incubated for 15 minutes. After removing the medium, the plates were placed on powdered dry ice to freeze the cells and then removed from dry ice. The cells were thawed with 50 µl of 50 mM HCl and frozen again on dry ice. The level of intracellular cAMP was measured with a commercially available cAMP EIA kit (Biotrack cAMP EIA system, GE Healthcare).

Stimulation of inositol phosphate. The stimulation of intracellular inositol phosphates (IPs) was measured in transiently transfected COS-7 cells that were pre-labeled (16 hours) with ^{3}H-myo-D-inositol (2 µCi/ml). Cells were treated with ligand in DMEM containing fetal bovine serum (10%) and

LiCl (30 mM) for 30 minutes; cells were lysed with ice cold trichloro acetic acid (5%) and IPs were extracted from the acid-lysates by ion-exchange filtration, as described (Shimizu et al., *J. Biol. Chem.* 276:49003-49012 (2001)).

OK cell methods. Cells were treated for 10 minutes at 37 °C with media (vehicle) or media containing a peptide ligand (1×10^{-7} M); then (t=0), the cells were then rinsed three times with media and incubated in alone at 37 °C for varying times. At each time point, $^{32}PO_4$ was then added to the media, and after five minutes of incubation, the cells were washed, lysed, and the lysate was counted for ^{32}P beta radioactivity by liquid scintillation counting. The results of these experiments are shown in Figure 11, plotted as a percentile of the amount of ^{32}P radioactivity in lysates of cells treated for the same time with vehicle alone.

Data calculations for in vitro binding and signaling assays. Data were processed for curve fitting and parameter determination using Microsoft Excel and GraphPad Prism 4.0 software packages. Dissociation time course data were analyzed using a bi-exponential decay equation, except when an F test analysis indicated a mono-exponential equation provided a better fit (*Palpha* > 0.02). Data from equilibrium binding, cAMP and IP dose-response assays were analyzed using a sigmoidal dose-response equation with variable slope. This analysis yielded curves for the data and values of EC_{50}, IC_{50} (the concentration of a ligand that produces half of the maximal effect) and E_{max} (the maximum response obtained by a ligand). Paired data sets were statistically compared using the Student's t-test (two-tailed) assuming unequal variances for the two sets.

Pharmacokinetic analysis of PTHrP(1-36) and I5-PTHrP(1-36) in normal rats. Concentration of human PTHrP(1-36) and [I^5]-PTHrP(1-36) in stock solution were adjusted by dilution with 25 mmol/L phosphate-citrate buffer/100 mmol/L NaCl/0.05% Tween 80 (pH.5.0) (PC-buffer). Both peptides were allowed to stand on ice immediately before administration.

Female SD-IGS rats at 8 weeks of age (Charles River Japan, Inc.) were measured for their body weight. Rats received intravenous administration of Human PTHrP(1-36) and [I^5]-PTHrP(1-36) at a dose of 10 nmol/1 ml/kg. Peptides were administered to groups of 3 rats for each peptide-dose and/or time point. At 2.5, 5, 7.5, 10, 15, 30, 60, 120 min after administration, blood was collected by tail vein in tubes with EDTA (final 0.2%) and aprotinin (final 0.6 TIU/ml) to monitor the time course of concentration of human PTHrP(1-36) and [I^5]-PTHrP(1-36) in rat plasma. Samples were centrifuged to collect plasma and stored at -80°C, until assayed for human PTHrP(1-36) and [I^5]-PTHrP(1-36) levels.

The level of human PTHrP(1-36) and [I^5]-PTHrP(1-36) were determined by EIA analysis using PTH-RP 1-34 (Human, Rat) Enzyme Immunoassay kit (Peninsula Laboratories Inc.) [I^5]-PTHrP(1-36) cross-reacted with PTHrP EIA kit, and [I^5]-PTHrP(1-36) was used as a standard for measurement of the level of [I^5]-PTHrP(1-36) in plasma.

Hypercalcemic action of human PTH(1-34), PTHrP(1-36) and PTH or PTHrP analogs in normal rats. Human PTH(1-34), PTHrP(1-36), and PTH or PTHrP analogs were studied for hypercalcemic effects in normal rat as follows. Concentration of peptides in stock solution were adjusted by dilution with 25 mmol/L phosphate-citrate buffer/100 mmol/L NaCl/0.05% Tween 80 (pH.5.0) (PC-buffer). All peptides were allowed to stand on ice immediately before administration.

Female SD-IGS rat at 8 weeks of age (Charles River Japan, Inc.) were measured for their body weight. Blood was collected by tail vein into heparinized capillary tubes and measured for baseline levels of blood ionized calcium and pH using Ca^{++}/pH analyzer (Model 634/Bayer Medical Ltd.) to give the corrected level of ionized calcium at pH 7.4 for each sample. Rats received intravenous administration of each peptides at a dose of 1 ml/kg. Peptides were administered to groups of 6 rats each respectively. At 1, 2, 4, or 6 hours after administration, blood was collected by tail vein to monitor the time course of corrected blood ionized calcium levels. The time course of

changes in corrected ionized calcium levels, compared to vehicle, and are expressed as means +/- standard error.

Statistical Analysis. Statistical analysis was carried out by analysis of variance (ANOVA), using SAS software. The significance of differences was determined using Student's t-test or Dunnett's multiple test. $P<0.05$ was considered a statistically significant.

Calcemic action of [$A^{1,3,12}$,Q^{10},R^{11},W^{14}]-hPTH(1-14)/PTHrP(15-36)(MPTH14) in thyroparathyroidectomy rats. Five-week-old male Crl:CD(SD) rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and acclimated for 1 week under standard laboratory conditions at 20-26 °C and 35-75% humidity. The rats received free access to tap water and standard rodent chow (CE-2) containing 1.1% calcium, 1.0% phosphate and 250 IU/100 g of vitamin D_3 (Clea Japan, Inc., Shizuoka, Japan).

Thyroparathyroidectomy (TPTX) was performed on six-week-old rats. TPTX rats were selected for use based on serum ionized calcium (iCa) levels (< 1.0 mM) in samples taken from tail vein bleeding at 24 hours or 72 hours after the operation using the electrode method. The TPTX rats were divided into six groups of five animals based on iCa levels at 48 hours after the operation. TPTX-vehicle group intravenously received the vehicle alone (10 mM acetic acid solution) at a dose of 1 ml/kg body weight administered to the tail vein. Human parathyroid hormone (1-34) (hPTH(1-34)) and M-PTH(1-14)/rP(15-36) (MPTH14) were intravenously injected into the TPTX rats at doses of 1.25, 5, 20 nmol/kg (3 groups) and 1.25, 5 nmol/kg (2 groups), respectively.

Blood was obtained from the tail vein for detecting iCa at 1, 2, 4, 6, and 24 hours after each injection. Ionized calcium levels were determined by the electrode method using an autoanalyzer (M-634, Chiba Corning Diagnostics Co. Ltd., Tokyo, Japan).

Mouse studies. Wild-type mice were injected subcutaneously with vehicle (0.9% NaCl/0.05% Tween-20), or vehicle containing a PTH peptide at a dose level of 10 to 1000 nmol/kg of body weight. At indicated times after

injection, blood was withdrawn from the tail vein, and the amount of cAMP in the resulting plasma was quantified by radioimmuno assay. Ionized calcium in serum was measured as above and phosphate was measured by a U.V. spectroscopic kit assay.

Statistical analysis for animal studies. Data are represented as the mean ± standard error (SE). Statistical significance was determined using SAS (Ver.5.00.010720, SAS Institute Japan, Tokyo, Japan). A p value of <0.05 was considered statistically significant. *P<0.05, **P<0.01, ***P<0.001 versus TPTX-vehicle level by Dunnett's multiple comparison test.

Example 2

Characterization of alanine substitutions in PTH and PTHrP

As shown above, PTH(1-34) has a greater capacity to bind to the R^0 receptor conformation than does PTHrP(1-36), which favors the RG conformation. To explore the molecular basis for this differential binding and conformational selectivity, we compared the effects of substitutions in the N-terminal and C-terminal regions of PTH and PTHrP peptides on the interaction of the ligands with the PTHR. Unlike in PTH(1-14), where alanine substitutions at positions 1, 3, 10, 11, 12 and 14 increased cAMP activity, each alanine substitution in PTHrP(1-14) abolished activity in cells expressing PTHR. Thus, the (1-14) regions of PTH and PTHrP interact with the juxtamembrane (J) region of the PTHR differently. Both PTHrP(1-14) and PTHrP(1-36) were much less potent for cAMP activity in cells expressing a PTHR lacking the extracellular N-terminal (N) domain (delNT), as compared to their respective PTH(1-14) and PTH(1-34) counterparts. PTHrP(1-36) activity therefore depends more heavily on interactions between the C-terminal ligand region and the PTHR N domain than does PTH(1-34) activity. We therefore studied the C-terminal region of the PTHrP sequence, as described in Example 3.

Example 3

C-terminal substitutions in PTH(1-28) and PTHrP(1-28)

Using alanine-scan and type-substitution strategies, we were able to generate peptides with much greater selectivity for RG receptor conformation than the native PTHrP(1-28) sequence. We focused our studies on the C-terminal region of the PTHrP sequence, and thus performed an alanine-scan of the 15-28 region of PTH(1-28) (data not shown) and PTHrP(1-28). Ala-scan analysis of the C-terminal regions of PTH(1-28) and PTHrP(1-28) revealed for each peptide strong reductions in activity at positions Arg^{20}, Trp/Phe^{23}, Leu^{24}, and Leu/Ile^{28}, known in PTH to form the core N domain-binding motif. Enhancements in activity were found at several, but different positions in each scaffold: Leu^{18}, Phe^{22}, and His^{26} in PTHrP(1-28) and Asn^{16}, Glu^{19}, and Ala^{22} in PTH(1-28). The alanine substitutions at positions 16, 19, and 22 in PTH increased binding to delNT (PTH receptor missing the N-terminal ligand binding domain), whereas those at positions 18, 22, 26 in PTHrP decreased binding to delNT. The enhancing effects of the Ala substitutions at positions 16, 19, and 22 of PTH are thus mediated via the PTHR J domain, whereas, those at positions 18, 22, 26 of PTHrP require the PTHR N domain. Further type substitution analysis of positions 16, 19, 22, as well as 25 (neutral to Ala substitution) in PTHrP(1-28) resulted in the analog $[Ala^{18,22},Leu^{25},Lys^{26}]$-PTHrP(1-28), which exhibits a cAMP potency and RG binding affinity that is greater than that of PTH(1-34) and among the highest observed of any PTH or PTHrP peptide. This scan revealed that alanine substitutions at positions 18, 22, 25, and 26 each enhance cAMP activity in human and rat PTHR-expressing cells (Figures 27A and 27B). Following the alanine scan, these positions were further substituted individually with various amino acids; of which some were found to increase cAMP activity (Figures 27C and 27D). We then combined these mutations in various combinations, thus obtaining a number of PTHrP analogs with markedly enhanced activity, as described herein.

Example 4

Characterization of exemplary substituted PTHrP(1-28) peptides

Dose-response curves for cAMP production in SaOS cells using PTHrP(1-36), PTHrP(1-28), $A^{18,22},K^{26}$-PTHrP(1-28), $A^{18,22},L^{25},K^{26}$ (AALK)-PTHrP(1-28), E^{18},A^{22},K^{26}-PTHrP(1-28), or $E^{18},A^{22},L^{25},K^{26}$ (EALK)-PTHrP(1-28) were generated (Figure 28A). Marked enhancements of cAMP-inducing activity were found for $A(E)^{18,22},L^{25},K^{26}$-PTHrP(1-28) (AALK or EALK), as compared to parental PTHrP(1-28).

These enhancing effects were confirmed in vivo studies (Figure 28B) by injecting C57BL/6 mice (3-month-old, male) intravenously with either vehicle, PTHrP(1-36), PTHrP(1-28), AALK-PTHrP(1-28), or EALK-PTHrP(1-28) (n = 3). Blood was withdrawn 10 minutes after injection and plasma level of cAMP was measured by RIA. Marked enhancements were also observed in the mouse assay for the AALK-PTHrP(1-28) and EALK-PTHrP(1-28) as compared to wt PTHrP(1-28). The greater apparent potency of PTHrP(1-36) peptide in these assays may reflect slower clearance of the longer-length peptide from the blood.

Example 5

Characterization of the RG selective peptide EALK-PTHrP(1-30)

We also characterized the effects of the EALK-PTHrP(1-30) peptide on cAMP production. Three month old male C57BL/6 mice were intravenously injected with either vehicle, rPTH(1-34), M-PTH(1-34) ($M=A^1,Aib^3,Q^{10},Har^{11},A^{12},W^{14},R^{19}$), or $E^{18},A^{22},L^{25},K^{26}$-(EALK)-PTHrP(1-30) (5 nmol/kg). In the cAMP experiment (Figure 29A), blood was withdrawn 10 minutes after injection and plasma level of cAMP was measured by RIA. In the calcium experiment (Figure 29B), blood was withdrawn prior to injection and 1, 2, 4, and 6 hours after injection. Ionized calcium was measured using a Ca^{++}/pH analyzer. The ligands induced approximately the same level of plasma cAMP, but the R^0 selective ligand, M-PTH(1-34) induced an ionized calcium response markedly more robust and more sustained than that of

PTH(1-34). By contrast, the RG-selective ligand, EALK-PTHrP(1-30) induced an ionized calcium response that was, similar, if not lower, than that of PTH(1-34).

A second set of experiments was performed in which mice received 5 nmol/kg intravenous daily treatment with rPTH(1-34), M-PTH(1-34), or EALK-PTHrP(1-30) for 14 days. Blood samples were taken at days 6 and 13, and markers of bone turnover (PINP, osteocalcin and CTX) were assessed by ELISA. The R^0 selective ligand, M-PTH(1-34) strongly induced increases in markers of both bone formation (PINP, Figures 30A and 30B; osteocalcin, Figure 30D) and bone resorption (CTX, Figures 30E and 30F), as early as day 6. By contrast, the RG-selective ligand, EALK-PTHrP(1-30) increased bone formation markers, with relatively smaller effects on the resorption marker, as evident on day 6 (Figures 30A, 30C, and 30E). Under the dose and time conditions analyzed, PTH(1-34) had only minor effects on bone markers.

Consistent with the effects on bone markers, M-PTH(1-34) robustly increased trabecular bone, but also detectably diminished cortical bone (Figure 31), consistent with its severe hypercalcemic actions (Figure 29B). By contrast, EALK-PTHrP(1-30) increased cortical bone thickness with significance in the distal femur (Figure 30 and Table xx), without inducing severe hypercalcemia. These findings demonstrate that the modified ligands with different R^0/RG selectivities have differential effects on bone metabolism. The findings also show that RG selective analogs, such as EALK-PTHrP(1-30), preferentially stimulate bone formation over bone resorption, and have beneficial effects on cortical bones with minimum effects on blood calcium levels. M-PTH(1-34) greatly increases the trabecular bone at the distal femur metaphysis, but induced cortical bone resorption at the mid-femur diaphysis, as indicated by erosion of endosteal surface.

Table 7 shows quatitation of bone structural parameters following two weeks of daily treatment of the above peptides. As described above, mice were treated intravenously) with either vehicle, rPTH(1-34), M-PTH(1-34), or EALK-PTHrP(1-30) daily for 14 days. All analogs significantly increased

bone mineral density at both femur and lumbar spine. Cortical wall thickness was significantly lower in both distal and mid femur region for M-PTH(1-34). In contrast, EALK-PTHrP(1-30) increased cortical bone thickness with significance in the distal femur.

Table 7. Bone structural parameters after two-week daily treatment in mice

	vehicle	PTH(1-34)	*P vs. veh*	M-PTH(1-34)	*P vs. veh*	EALK-PTHrP (1-30)	*P vs. veh*
Piximus[a]							
Total Femur BMD (g/cm^2)	0.0599 ± 0.0002	0.0615 ± 0.0003	0.003	0.664 ± 0.0003	<0.0001	0.620 ± 0.0003	0.0004
Lumbar Spine BMD (g/cm^2)	0.0455 ± 0.0001	0.0464 ± 0.0002	0.001	0.0524 ± 0.0002	<0.0001	0.0464 ± 0.0002	0.001
microCT[b]							
distal femur							
TrabecularBV/TV (%)	17.6 ± 0.8	17.4 ± 1.2	0.883	35.0 ± 3.0	0.001	16.5 ± 1.3	0.506
Tb.N (1/mm)	4.37 ± 0.08	4.02 ± 0.14	0.055	5.22 ± 0.34	0.047	4.13 ± 0.16	0.201
Tb.Th (µm)	55.4 ± 1.7	57.5 ± 1.4	0.361	71.5 ± 1.8	<0.0001	57.8 ± 2.1	0.393
TbSp (µm)	224 ± 5	246 ± 10	0.076	208 ± 17	0.398	238 ± 10	0.235
Conn-Dens. ($1/mm^3$)	132 ± 4	116 ± 8	0.091	263 ± 25	0.002	117 ± 9	0.132
Cort Th. (µm)	213 ± 7	229 ± 11	0.229	166 ± 6	0.0003	238 ± 9	0.048
mid femur							
TA (mm^2)	2.05 ± 0.07	2.17 ± 0.05	0.197	2.10 ± 0.06	0.630	2.03 ± 0.06	0.840
BA(mm^2)	0.813 ± 0.019	0.839 ± 0.032	0.503	0.837 ± 0.022	0.423	0.821 ± 0.027	0.825
MA (mm^2)	1.24 ± 0.05	1.33 ± 0.02	0.177	1.26 ± 0.04	0.792	1.21 ± 0.03	0.702
BA/TA (%)	39.7 ± 0.9	38.7 ± 0.7	0.380	40.0 ± 0.8	0.837	40.3 ± 0.6	0.614
Cort Th. (µm)	172 ± 4	172 ± 5	0.965	151 ± 3	0.003	176 ± 4	0.558

Example 6

Optimization of EALK-PTHrP peptides

To optimize the activity of the EALK-PTHrP peptides, we generated EALK-PTHrP(1-30) and PTHrP(1-34) variants with substitutions in the 29-33 region. In the 1-30 scaffold, Gly, Ser, Leu, Asn, Gln, Trp, Glu, and Lys were substituted at position 29; Gly, Ser, Leu, Asn, Asp, Trp, and Lys were substituted at position 30; and Ser, Leu, Asn, Val, Trp, Glu, and Lys were substituted at position 31. In EALK-PTHrP(1-34), the 30-33 region was substituted with alanine, or the C-terminal six amino acids were replaced by the corresponding region of PTH(1-34). A predicted advantage of these longer-length peptides, relative to the PTHrP(1-30) scaffold, is that they will have longer a longer half-life in circulation due to slower clearance. The C-terminal substitutions were thus designed to provide the added chain length, but to avoid increasing R^0 binding affinity, which occurs when the native PTHrP(29-34)

region is installed. These peptides were tested for cAMP activity in MC3T3-E1 cells. As shown in Figures 32A and 32B, several of these peptides exhibited greater activity than the unsubstituted C-terminal sequence.

Example 7

Characterization of Trp^1-M-PTH in renal phosphate transport

To help elucidate further the signaling mechanisms by which PTH ligands regulate renal phosphate transport, we developed a derivative of M-PTH(1-28) that is defective for PLC/PKC signaling, yet retains potent cAMP/PKA signaling activity. Such a peptide allows for study of the relative roles of the PKA and PKC signaling pathways in modulating the function and surface expression of the Pi transporters NaPi-IIa and NaPi-IIc in proximal tubule (PT) cells. The analog M-PTH(1-28) ($M = Ala^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19}$), a potent agonist for cAMP and IP_3 signaling pathways, induces, when injected into mice, prolonged hypophosphatemic and hypercalcemic effects. The analog also induced prolonged reductions in NaPi-IIa immunoreactivity at the brush border membrane and cytoplasmic compartments of renal PT cells of injected mice.

To impair PLC signaling, we replaced alanine at position 1 of M-PTH(1-28) with tryptophan, in accordance with findings of Bisello and colleagues (J Biol Chem 277:38524-30, 2002) showing that such bulky substitutions at this position selectively impair PLC signaling. In HEK-293 cells transiently transfected with the rat PTHR, Trp^1-M-PTH(1-28) was about as potent as M-PTH(1-28) for stimulating cAMP formation, but at least 100-fold less potent than the parent peptide for stimulating IP_3 formation. Trp^1-M-PTH(1-28) retained the capacity to produce a prolonged cAMP response in MC3T3-E1 cells after ligand wash-out, as seen with MPTH(1-28). When injected into mice (20 nmol/kg) Trp^1-M-PTH(1-28), like M-PTH(1-28), induced prolonged suppression of plasma phosphate levels, as compared to effects of PTH(1-34): maximal suppression at 2h for each analog; recovery to vehicle control levels at 4h for PTH(1-34), and at 6h for M-PTH(1-28) and

Trp^1-M-PTH(1-28). Apical and cytoplasmic NaPi-IIa staining in renal PT cells was reduced in mice treated with each peptide at 2h, but where staining returned to vehicle control levels at 6h with PTH(1-34), it remained reduced for at least six hours in mice treated with M-PTH(1-28) or Trp^1-M-PTH(1-28). Immunostaining of NaPi-IIc in renal PT cells was reduced in mice treated with M-PTH(1-28) over the interval 4 to 6 h, but was unchanged in mice treated with Trp^1-M-PTH(1-28) or PTH(1-34). M-PTH(1-28) inhibited ^{32}P uptake in early passage LLC-PK1 cells (NHERF-1/ezrin positive) virally transduced to express NaPi-IIc transporter and the rat PTHR (Mahon, *Am J Physiol Renal Physiol.* 294:F667-75 (2008)), but Trp^1-M-PTH(1-28) failed to inhibit this activity. The findings suggest that PTHR-mediated regulation of renal Pi transport involves, as one component, the cAMP/PKA-dependent control of NaPi-IIa down regulation, and, as another, perhaps slower and minor component, the PLC-dependent control of NaPi-IIc down regulation.

Example 8

Characterization of M-PTH(1-14)/PTHrP(15-36) on serum and urinary calcium and phosphate in TPTX rats.

We also studied the effects of the M-PTH(1-14)/PTHrP(15-36) hybrid peptide (SP-PTH) on serum and urinary calcium and phosphate. A single intravenous injection into thyroparathyroidectomized (TPTX) rats, PTH(1-34) at 1.25 nmol/kg, transiently increased serum calcium(sCa) and decreased serum phosphorus (sPi) levels at 1 hr, but not to the normal range, as levels returned to pre-injection conditions by 6 hrs (Figures 33 and 35, respectively). PTH(1-34) did not change urinary calcium (Figure 34) or urinary phosphate levels (Figure 36) over 0-6 hours. By contrast, administration of SP-PTH at 1.25 nmol/kg, increased sCa and decreased sPi to normal levels within 6 hrs, and these levels were maintained for 24 hrs. SP-PTH decreased urinary calcium and increased urinary phosphate level at 0-6 hours. These results indicate that SP-PTH can normalize hypocalcemia in TPTX rats without causing

hypercalciuria, thus suggesting that this peptide can be used to treat hypoparathyroidism with decreased risk of renal complications.

Example 9

cAMP stimulation using PTH or PTHrP analogs

HKRK-B, which are LLC-PK1 cells over-expressing human PTH1 receptor at levels of 9.5 x 10^5 per cell were used in the cAMP signaling assay. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen Corp). Human PTHrP(1-36) was synthesized by American Peptide Company, Inc. (California, USA), Human PTH(1-34) was purchased from Peptide Institute Inc. (Osaka, Japan), and the PTH or PTHrP analogs (Mc-PTH(1-34), [A^1,A^3,A^{23},Q^{10},R^{11}]-hPTH(1-34), [A^1,A^3,A^{23}]-hPTH(1-34), and [A^{18},A^{22},L^{25},K^{26}]-PTHrP(1-28)) were synthesized by Sigma Aldrich Japan (Tokyo, Japan). All peptides were dissolved at 1 mM in 10 mM acetic acid, and stored at -80 °C. The cAMP stimulation assay was performed as described above for HKRK-B7 cells. PTH(1-34) and PTHrP(1-36) were used as controls. Cells were treated for 15 minutes at 37 °C with varying concentrations of ligands in the presence of IBMX. The EC_{50} and Emax values are reported in Table 8. All M-modified PTH analogs with C-terminal modification show comparable cAMP signaling to hPTH(1-34) (Figure 37).

Table 8.

	cAMP in HKRK-B7 cells	
	EC50 (nM)	Max (pm/well)
hPTH(1-34)	2.26	67.2
PTHrP(1-36)	1.47	61.9
Mc-PTH34(R19)	3.25	65.5
[A1,3,23,Q10,R11]-hPTH(1-34)	1.76	63.8
[A1,3,23]-hPTH(1-34)	1.93	66.6
[A18,22,L25,K26]-PTHrP(1-28)	0.52	56.4

Example 10

Use of short-acting PTH peptides for treatment of osteoporosis

Short-acting peptides, such as those described above, are administered to a patient having osteoporosis. Generally, in the case of the therapy of osteoporosis by intermittent i.v./i.m. or subcutaneous injection, the dosage given is in the range of 100 to 1200 units (μg)/day.

The exact doses and regimen for administration of these compounds and compositions will necessarily be dependent upon the needs of the individual subject being treated, the type of treatment, the degree of affliction or need and, of course, the judgment of the medical practitioner. In general, parenteral administration requires lower dosage than other methods of administration which are more dependent upon absorption.

Example 11

Use of long-acting PTH peptides for treatment of PTH deficiency

Long-acting peptides, such as those described above, are administered to a patient having a disease linked to PTH deficiency. Examples of these diseases include hyperphosphatemia associated with tumoral calcinosis, early stage chronic kidney disease and hypoparathyroidism. The daily dosage of peptide to be administered depends upon the indication. Generally, in the case

of daily i.v./i.m. or subcutaneous injection preferably at 300-2400 units (μg)/day.

The exact doses and regimen for administration of these compounds and compositions will necessarily be dependent upon the needs of the individual subject being treated, the type of treatment, the degree of affliction or need and, of course, the judgment of the medical practitioner. In general, parenteral administration requires lower dosage than other methods of administration, which are more dependent upon absorption.

Other Embodiments

All patents, patent applications, and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent, patent application, or publication was specifically and individually indicated to be incorporated by reference. U.S. Provisional Application Nos. 60/963,117, 60/963,082, and 60/963,867, filed August 1, 2007, August 2, 2007, and August 6, 2007, respectively, are hereby incorporated by reference.

What is claimed is:

CLAIMS

1. A method for determining whether a candidate compound is a long-acting agonist of a G protein coupled receptor (GPCR), said method comprising:

(a) contacting said GPCR with said compound, wherein said GPCR is in the RG form;

(b) measuring the affinity of said compound for the RG form of said GPCR;

(c) contacting said GPCR with said compound, wherein said GPCR is in the R^0 form; and

(d) measuring the affinity of said compound for the R^0 form of said GPCR, wherein a compound that (i) that has an affinity for the RG form of said GPCR that is at least 10% of an endogenous agonist for said GPCR, and (ii) has a greater affinity for the R^0 form of said GPCR than said endogenous agonist is identified as a long-acting agonist of said GPCR.

2. The method of claim 1, further comprising the steps of:

(e) administering said candidate compound to an animal, and

(f) measuring at least one physiological response of said animal to said compound.

3. The method of claim 1, wherein said receptor is a human receptor.

4. The method of claim 1, wherein said GPCR is a secretin family receptor.

5. The method of claim 4, wherein said receptor is a PTH/PTHrP receptor.

6. The method of claim 5, wherein said PTH/PTHrP receptor is a human receptor.

7. The method of claim 5, wherein said measuring step (b) is performed by measuring intracellular or blood calcium levels.

8. The method of claim 1, wherein said measuring step (b) or step (d) is performed using a competition binding assay.

9. The method of claim 8, wherein said competition binding assay uses a ligand that is specific for the RG form or specific for the R^0 form of the GPCR.

10. The method of claim 1, wherein said measuring step (b) is performed using a delayed cAMP assay.

11. The method of claim 1, wherein said R^0 form of said GPCR is enriched using a nonhydrolizable nucleotide analog.

12. The method of claim 11, wherein said nucleotide analog is GTPγS.

13. The method of claim 1, wherein said RG form of said GPCR is enriched using a dominant-negative G-protein.

14. The method of claim 1, wherein said receptor is on a cell or in a membrane.

15. The method of claim 1, wherein said candidate compound comprises a peptide.

16. The method of claim 1, wherein said candidate compound is from a chemical library or natural product library.

17. A method for determining whether a candidate compound is a short-acting agonist of a G protein coupled receptor (GPCR), said method comprising:

(a) contacting said GPCR with said compound, wherein said GPCR is in the RG form;

(b) measuring the affinity of said compound for the RG form of said GPCR;

(c) contacting said GPCR with said compound, wherein said GPCR is in the R^0 form; and

(d) measuring the affinity of said compound for the R^0 form of said GPCR, wherein a compound that (i) that has an affinity for the RG form of said GPCR that is at least 10% of an endogenous agonist for said GPCR, and (ii) has a lower affinity for the R^0 form of said GPCR than said endogenous agonist is identified as a short-acting agonist of said GPCR.

18. The method of claim 17, wherein said receptor is a human receptor.

19. The method of claim 17, further comprising the steps of:

(e) administering said candidate compound to an animal, and

(f) measuring at least one physiological response of said animal to said compound.

20. The method of claim 16, wherein said GPCR is a secretin family receptor.

21. The method of claim 20, wherein said receptor is a PTH/PTHrP receptor.

22. The method of claim 21, wherein said PTH/PTHrP receptor is a human receptor.

23. The method of claim 21, wherein said measuring step (b) is performed by measuring intracellular or blood calcium levels.

24. The method of claim 17, wherein said measuring step (b) or step (d) is performed using a competition binding assay.

25. The method of claim 23, wherein said competition binding assay uses a ligand that is specific for the RG form or specific for the R^0 form of the GPCR.

26. The method of claim 17, wherein said measuring step (b) is performed using a delayed cAMP assay.

27. The method of claim 17, wherein said R^0 form of said GPCR is enriched using a nonhydrolizable nucleotide analog.

28. The method of claim 11, wherein said nucleotide analog is GTPγS.

29. The method of claim 17, wherein said RG form of said GPCR is enriched using a dominant-negative G-protein.

30. The method of claim 17, wherein said receptor is on a cell or in a membrane.

31. The method of claim 17, wherein said candidate compound comprises a peptide.

32. The method of claim 17, wherein said candidate compound is from a chemical library or a natural product library.

33. A polypeptide having affinity for PTH RG and a low affinity for PTH R^0.

34. The polypeptide of claim 33, having an amino acid sequence modified by a substitution, deletion, and/or addition of one or more amino acids relative to the wild-type PTH or PTHrP sequence.

35. The polypeptide of claim 34, wherein the amino acid has a histidine at position 5 or an alanine at position 23.

36. The polypeptide of claim 33 having at least one the amino acid substitutions as compared to the wild-type sequence in the peptides represented by the following formulas Ala^{23}PTH, His^5-PTH, and His^5-PTHrP, or a fragment thereof.

37. The polypeptide of claim 34, wherein the amino acid sequence has a lower affinity for an amino-terminal extracellular domain of the PTHR as compared to the wild-type PTH.

38. The polypeptide of claim 37, wherein the amino acid sequence comprises at least one substitution selected from the group consisting of alanine at position 20, alanine at position 23, alanine at position 24, and alanine at position 28 of the PTH or PTHrP sequence.

39. The polypeptide of claim 33 selected from the group consisting of any of those identified as RG selective in the table of Figure 26B.

40. The polypeptide of claim 34 or 37 comprising the amino acid sequence:

X1-Val-X2-Glu-His-Gln-Lys-Met-His-X3-X4-X5-X6-X7,

wherein:

X1 is Ser, Ala, Gly, or Aib;

X2 is Ser, Ala, or Aib;

X3 is Ala, Asn, Glu, Val, Asp, or Gln;

X4 is Leu, Val, Ala, Trp, Ile, Met, Lys, Arg, or Har;

X5 is Gly, His, Arg, Ala, or Aib;

X6 is Lys, Gln, Leu, His, Trp, Ala, Arg, or Aib; and

X7 is His, Arg, Leu, Phe, Trp, or Aib, or a fragment thereof containing amino acids 1-10, 1-11, 1-12, or 1-13, or a pharmaceutically acceptable salt thereof.

41. The polypeptide of claim 40, wherein said polypeptide has an amino acid sequence fewer than 20 amino acids in length.

42. The polypeptide of claim 41, wherein said amino acid sequence is 10 to 14 amino acids in length.

43. The polypeptide of claim 40, consisting essentially of said amino acid sequence, or said fragment thereof.

44. The polypeptide of claim 43, consisting of said amino acid sequence, or said fragment thereof.

45. The polypeptide of claim 33, comprising the amino acid sequence:

X1-Val-X2-Glu-X3-Gln-Leu-Met-His-X4-X5-X6-X7-X8-Leu-Asn-Ser-Met-Glu-X9-Val-Glu-X10-X11-Arg-Lys-Lys-X12,

wherein:

X1 is Ser, Ala, or Aib;

X2 is Ser, Ala, or Aib;

X3 is Ile or His;

X4 is Asn, Glu, Val, Asp, Glu, or Gln;

X5 is Leu, Val, Ala, Trp, Ile, Met, Lys, Arg, or Har;

X6 is Gly, His, Arg, or Ala;

X7 is Lys, Gln, Leu, His, Trp, Ala, or Arg;

X8 is His, Arg, Leu, Phe, or Trp;

X9 is Arg or Ala;

X10 is Trp, Phe, or Ala;

X11 is Leu or Ala; and

X12 is Leu or Ala;

a fragment thereof comprising amino acids 1-24, 1-25, 1-26, or 1-27 of said amino acid sequence, or a pharmaceutically salt thereof, wherein the amino acid sequence comprises at least one of amino acids selected from the group consisting of His at position X3, Ala at position X9, Ala at position X10, Ala at position X11, and Ala at position X12.

46. The polypeptide of claim 45, wherein said polypeptide is fewer than 50 amino acids in length.

47. The polypeptide of claim 46, wherein said polypeptide is 24-28 amino acids in length.

48. The polypeptide of claim 45, consisting essentially of said amino acid sequence, or said fragment thereof.

49. The polypeptide of claim 48, consisting of said amino acid sequence, or said fragment thereof.

50. The polypeptide of claims 45-49 wherein at least one of X9, X10, X11, or X12 is alanine.

51. A composition comprising a polypeptide of claims 33-50 and a pharmaceutically acceptable carrier.

52. A method for treating osteoporosis in a subject comprising administering the polypeptide or composition of any of claims 33-51 to said subject in need thereof in an amount sufficient to treat osteoporosis.

53. A method for treating fracture repair, osteomalacia, arthritis, thrombocytopenia, hypoparathyroidism or hyperphosphatemia or increasing stem cell mobilization in a subject, comprising administering the polypeptide of any of claims 33-51 to said subject in an amount sufficient to treat said disease or to increase stem cell mobilization.

54. The method according to claim 52 or 53 wherein the route of administration is selected from the group consisting of subcutaneously, intravenously, intranasally, transpulmonarily, transdermally, and orally.

55. A polypeptide which binds the PTH receptor and has a high affinity for the R^0 form of the PTH receptor.

56. The polypeptide of claim 55, having an amino acid sequence modified by a substitution, deletion and/or addition of one or more amino acids relative to the wild-type PTH or PTHrP sequence.

57. The polypeptide of claim 55, selected from the group consisting of any of the peptides of Figure 26B having an IC_{50} less than 7.9 nM, Ala^1,Aib^3[M]PTH(1-28), Ala^1,Aib^3[M]PTH(1-34), and I^5-hPTHrP(1-36) (#1208).

58. The polypeptide of claim 55, wherein the amino acid sequence has a higher affinity for the amino-terminal extracellular domain of the PTHR as compared to wild-type PTH.

59. The polypeptide of claim 55 or 58, wherein the amino acid sequence comprises arginine at position 19 or isoleucine at position 5.

60. The polypeptide of claims 55-59,comprising the amino acid sequence of the formula:

X1-Val-X2-Glu-Ile-Gln-Leu-Met-His-X3-X4-X5-X6-X7-Leu-Asn-Ser-Met-Arg-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu,

wherein:

X1 is Ser, Ala, or Aib;

X2 is Ser, Ala, or Aib;

X3 is Asn, Glu, Val, Asp, Glu, or Gln;

X4 is Leu, Val, Ala, Trp, Ile, Met, Lys, or Arg;

X5 is Gly, His, Arg, or Ala;

X6 is Lys, Gln, Leu, His, Trp, Ala, or Arg; and

X7 is His, Arg, Leu, Phe, or Trp,

or a fragment thereof containing amino acids 1-24, 1-25, 1-26, or 1-27 of said amino acid sequence, or a pharmaceutically acceptable salt thereof.

61. The polypeptide of claim 60, wherein said polypeptide is fewer than 50 amino acids in length.

62. The polypeptide of claim 61, wherein said polypeptide is 24-28 amino acids in length.

63. The polypeptide of claim 60, wherein said polypeptide consists essentially of said amino acid sequence, or a fragment thereof.

64. A pharmaceutical composition comprising a polypeptide of claims 60-63 and a pharmaceutically acceptable carrier.

65. A method for treating disease or condition selected from the group consisting of hypoparathyroidism, hyperphosphatemia, tumoral calcinosis, and osteoporosis in a subject, said method comprising administering the polypeptide of any of claims 55-57 to a subject in need thereof in an amount sufficient to treat said disease or condition.

66. A method for treating a subject needing fracture repair, or having osteomalacia, arthritis, thrombocytopenia, or requiring stem cell mobilization comprising administering the polypeptide of any of claims 55-57 to said subject in an amount sufficient to repair said fracture, to treat said disease, or to mobilize stem cells.

67. The method according to claim 65 or 66 wherein the route of administration is selected from the group consisting of subcutaneously, intravenously, intranasally, transpulmonarily, transdermally, and orally.

68. A polypeptide comprising the amino acid sequence:
Ala-Val-Ser-Glu-His-Glu-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-X1-Arg-Arg-Arg-X2-Phe-Leu-X3-X4-Leu-Ile-X5-X6-X7-X8-X9-X10-Glu-Ile

wherein:

X1 is Leu, Ala, Ser, Met, Phe, or Glu;

X2 is Phe, Ala, Ser, Leu, Asn, Trp, Glu, or Lys;

X3 is His, Leu, Arg, Lys, Trp, Ile, or Phe;

X4 is His, Ala, Ser, Asn, Lys, or Arg;

X5 is Ala, Gly, Ser, Asn, Gln, Trp, Glu, or Lys;

X6 is Glu, Gly, Ser, Leu, Asn, Asp, Lys, or Ala;

X7 is Ile, Leu, Val, Lys, or Ala;

X8 is His or Ala

X9 is Thr, Asn, or Ala; and

X10 is Ala or Phe,

or a fragment thereof containing amino acids 1-24, 1-25, 1-26, 1-27, 1-28, 1-29, 1-30, 1-31, 1-32, 1-33, 1-34, or 1-35 of said amino acid sequence, and wherein said polypeptide comprises at least one amino acid substitution as compared to the corresponding wild type PTHrP sequence or a fragment thereof; or a pharmaceutically acceptable salt thereof.

69. The polypeptide of claim 68, wherein said polypeptide is fewer than 50 amino acids in length.

70. The polypeptide of claim 69, wherein said polypeptide or fragment thereof is 28 to 36 amino acids in length.

71. A polypeptide of claims 68-70 having at least one amino acid substitution selected from the group consisting of X1 is Ala or Glu, X2 is Ala, X3 is Leu, and X4 is Lys.

72. The polypeptide of claims 68-70, wherein said polypeptide comprises an amino acid sequence shown in Table 1.

73. The polypeptide of claim 71 or 72 comprising the amino acid sequence [Ala^{18},Ala^{22},Leu^{25},Lys^{26}]-PTHrP(1-28) or [Glu^{18},Ala^{22},Leu^{25},Lys^{26}]-PTHrP(1-28).

74. The polypeptide of claim 73 consisting of the amino acid sequence [Ala^{18},Ala^{22},Leu^{25},Lys^{26}]-PTHrP(1-28) or [Glu^{18},Ala^{22},Leu^{25},Lys^{26}]-PTHrP(1-28).

75. The polypeptide of claims 68-70, wherein said polypeptide has a hydroxyl group or is amidated at its C-terminus.

76. The polypeptide of claims 68-75, wherein said polypeptide consists essentially of said amino acid sequence or a fragment thereof.

77. The polypeptide of claims 68-76, wherein said polypeptide is RG selective or has a low binding affinity to the R^0 form of the PTH receptor.

78. A pharmaceutical composition comprising any of the polypeptides of claims 68-77 and a pharmaceutically acceptable carrier.

79. A method for treating osteoporosis in a subject comprising administering the polypeptide or composition of any of claims 68-78 to said subject in need thereof in an amount sufficient to treat osteoporosis.

80. A method for treating fracture repair, osteomalacia, arthritis, thrombocytopenia, hypoparathyroidism or hyperphosphatemia or increasing stem cell mobilization in a subject, comprising administering the polypeptide of any of claims 68-78 to said subject in an amount sufficient to treat said disease or to increase stem cell mobilization.

81. The method according to claim 79 or 80 wherein the route of administration is selected from the group consisting of subcutaneously, intravenously, intranasally, transpulmonarily, transdermally, and orally.

82. A nucleic acid comprising a sequence encoding a polypeptide of any of claims 33-50, 55-63, and 68-77.

83. The nucleic acid of claim 82 operably linked to a promoter.

84. A vector comprising a nucleic acid of claim 83.

85. A cell comprising the vector of claim 84.

86. A method of making a polypeptide comprising growing the cell of claim 85 under conditions where said polypeptide is expressed.

Figure 1A

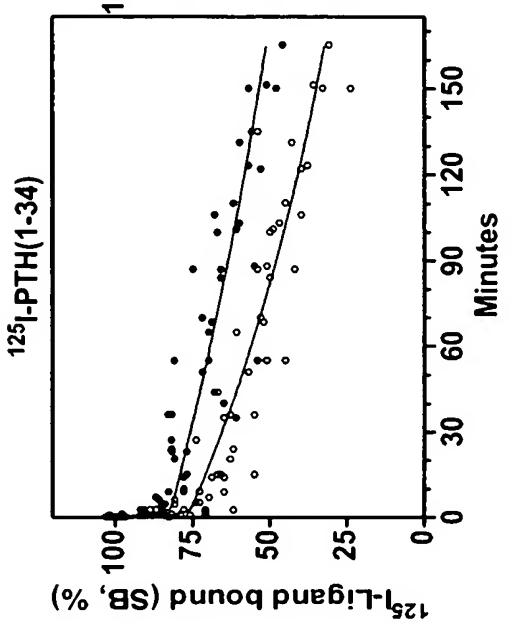


Figure 1B

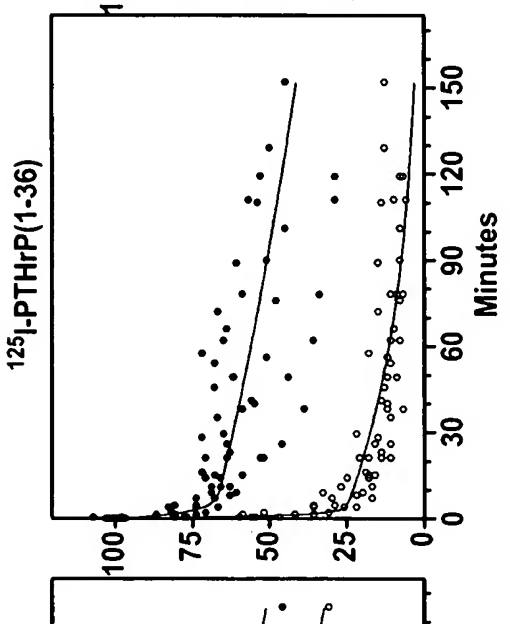


Figure 1C

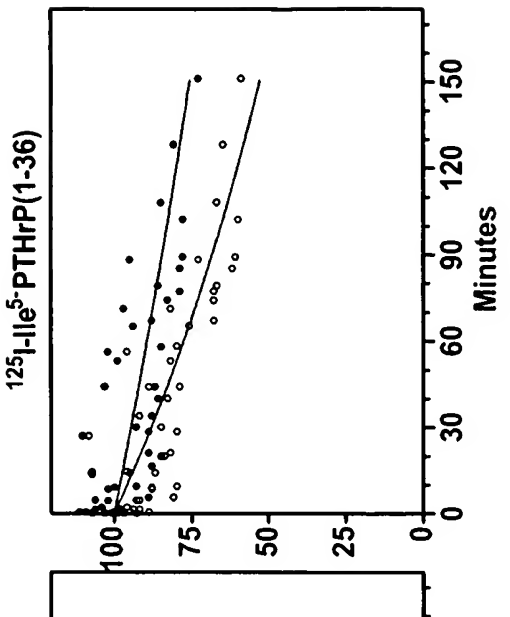


Figure 2A

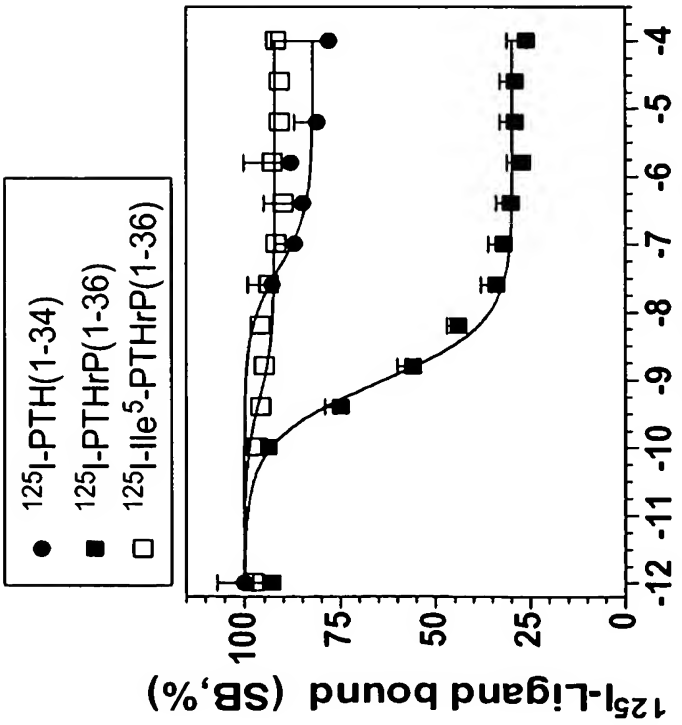
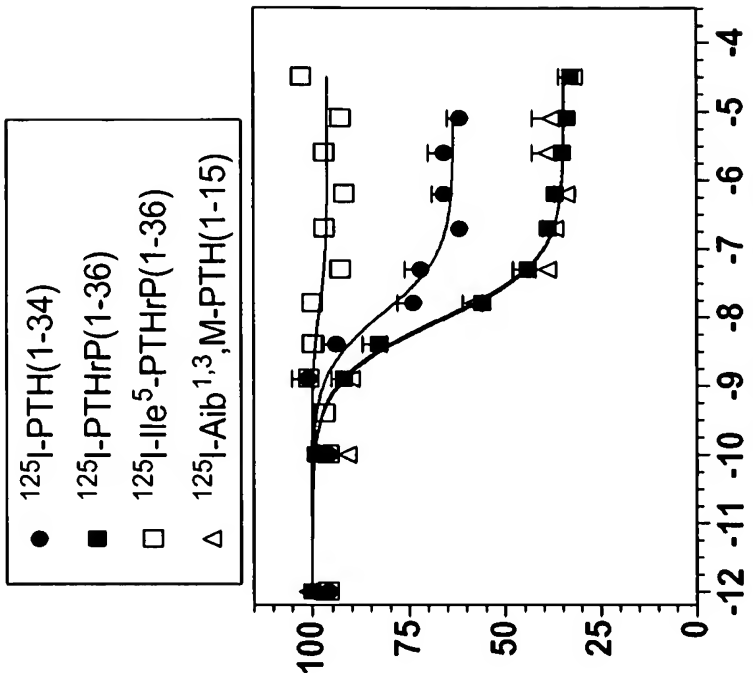
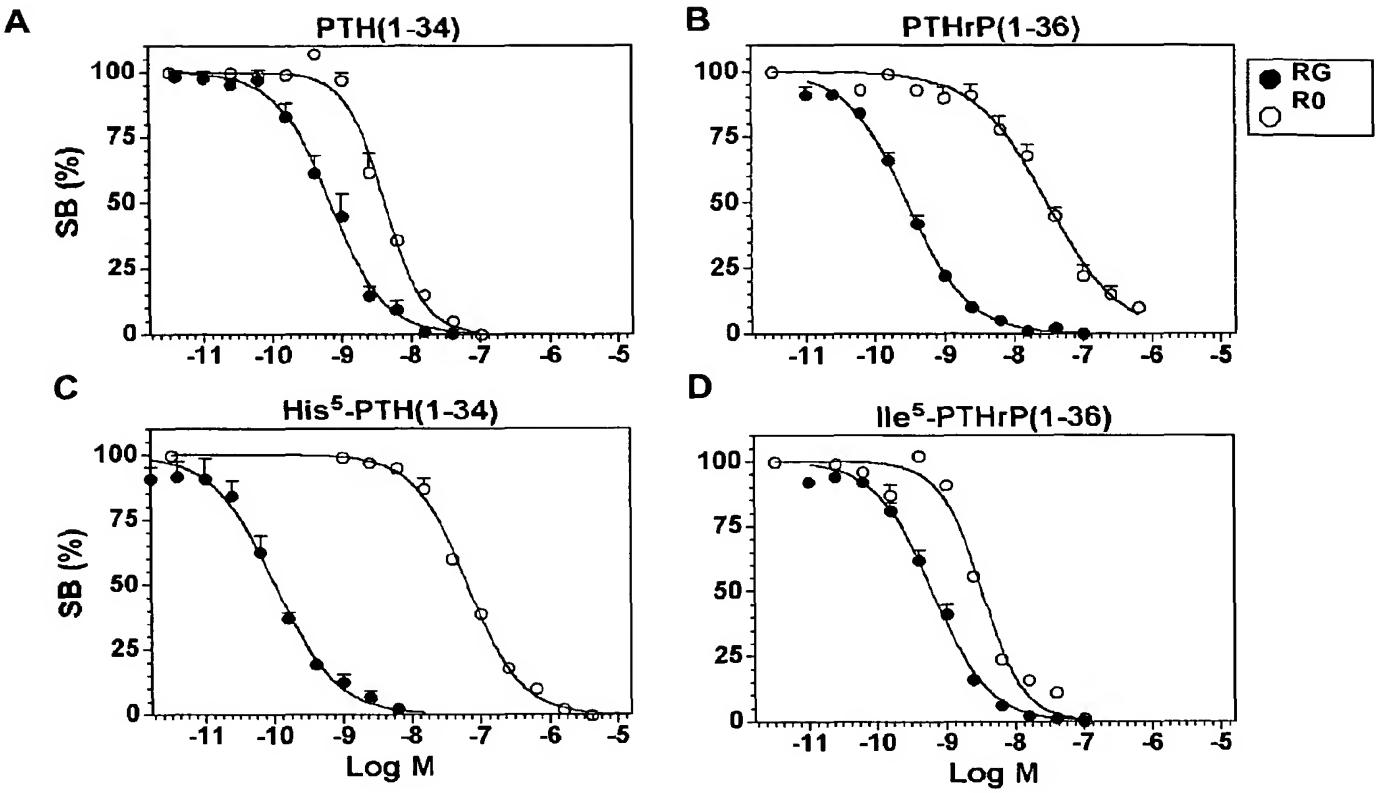
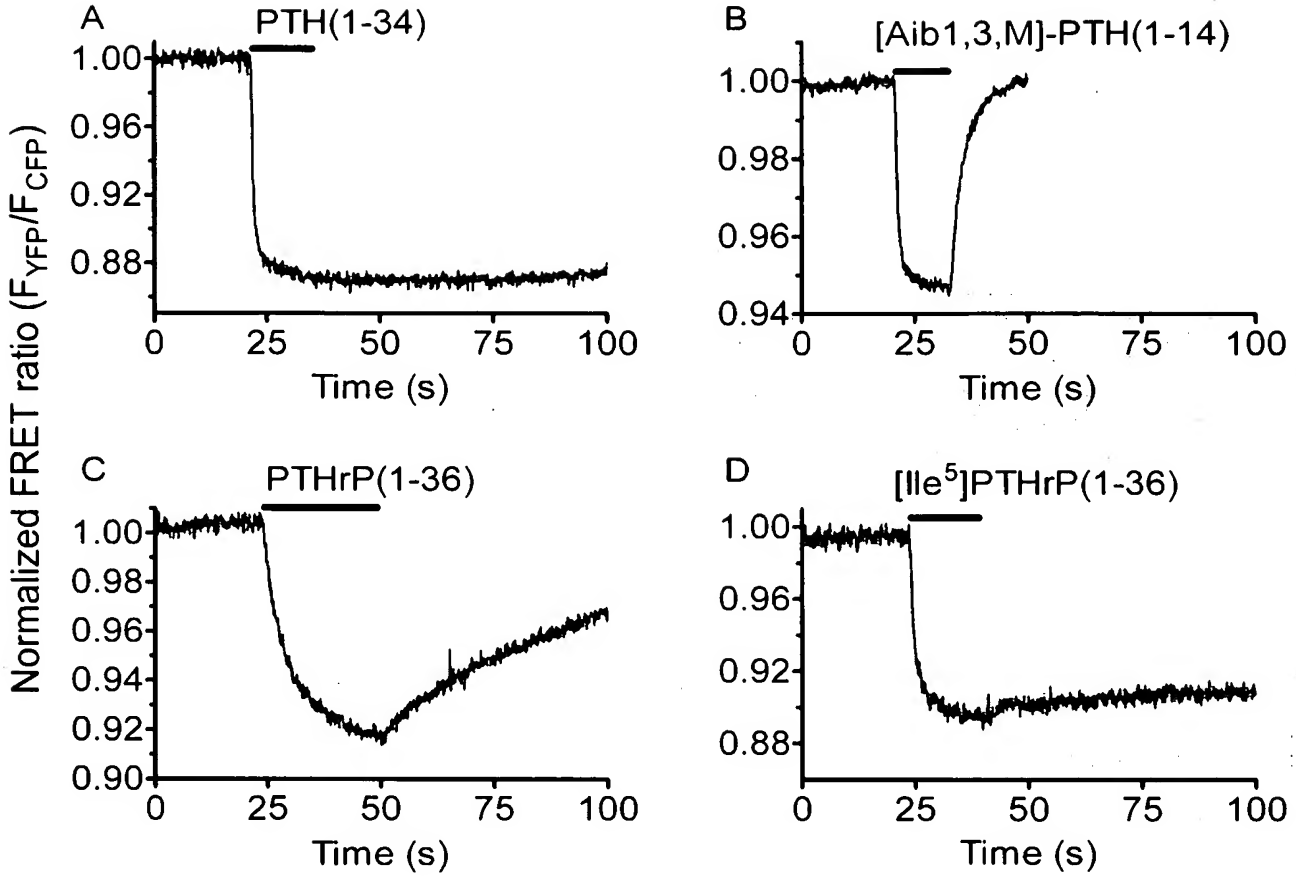


Figure 2B





Figures 3A-3D



Figures 4A-4D

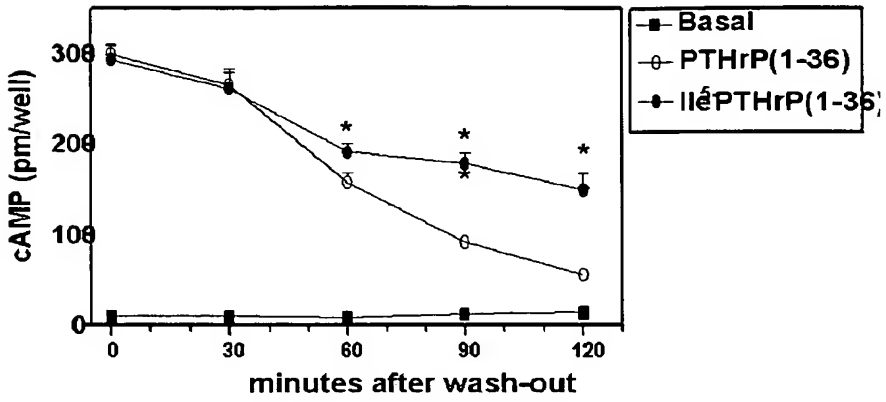
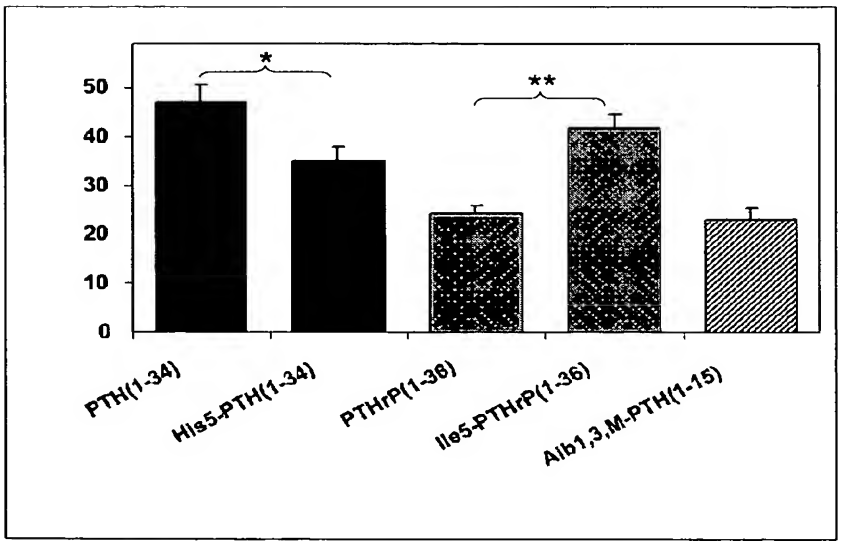
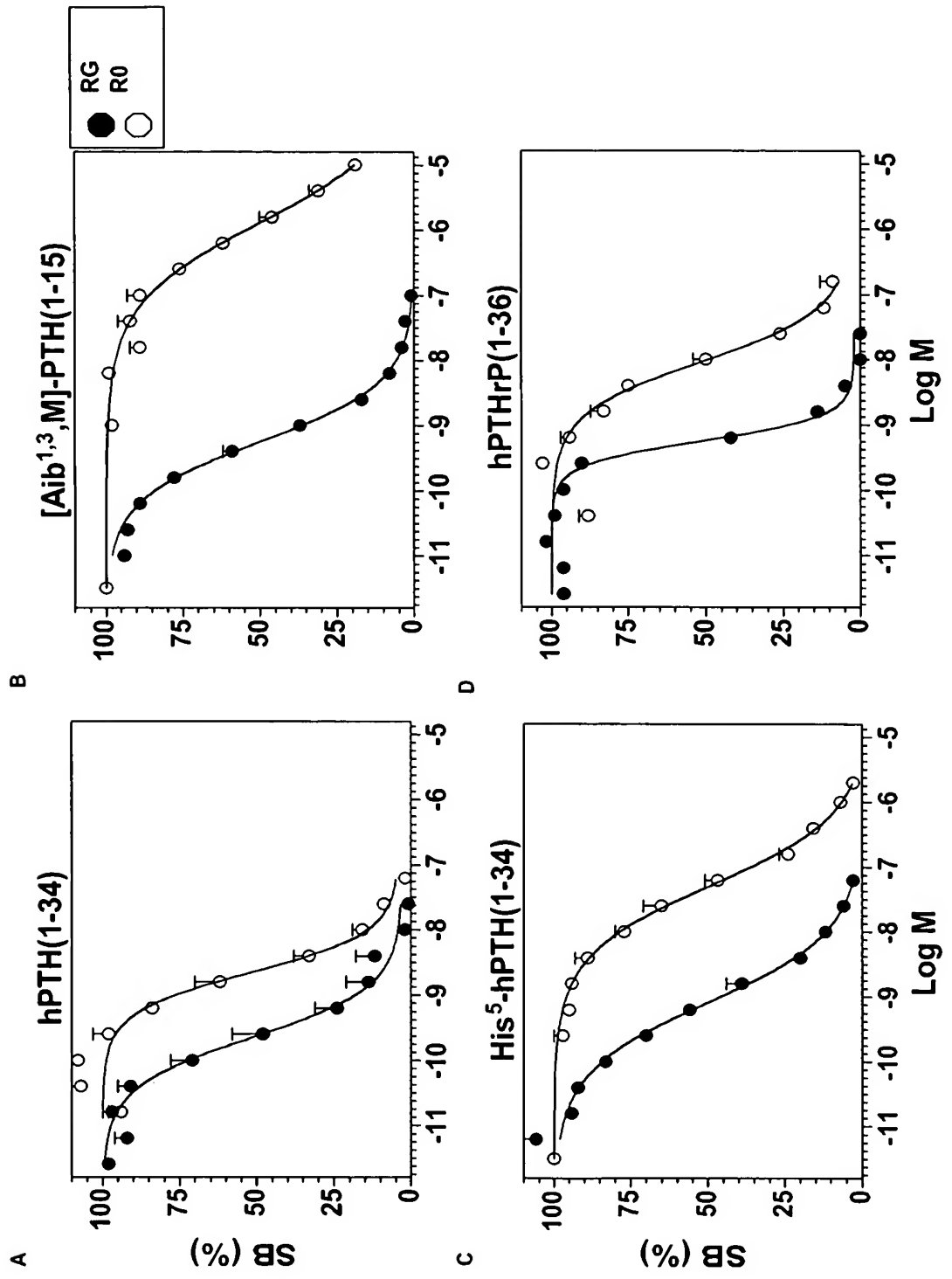


Figure 5A

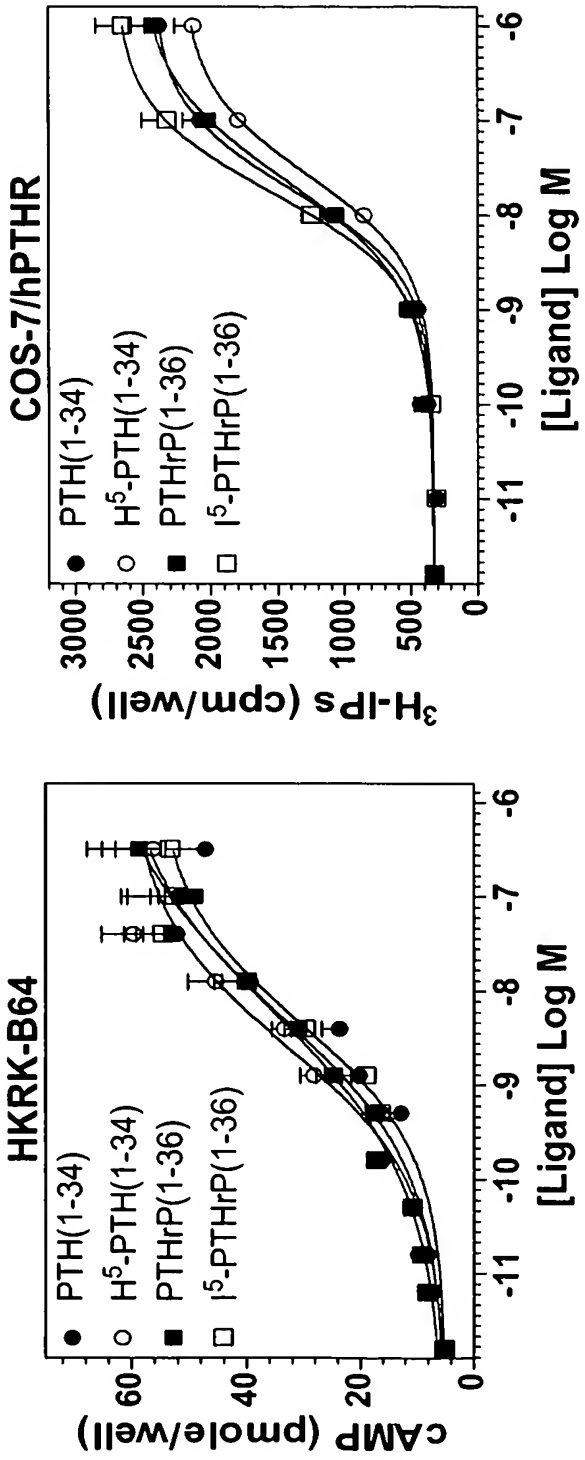


	maximum cAMP pmole/well			*P* vs. PTH(1-34)
basal	6.4	±	1.9	
PTH(1-34)	157	±	10	
His^5-PTH(1-34)	158	±	8	1.0
PTHrP(1-36)	148	±	11	0.6
Ile^5-PTHrP(1-36)	147	±	10	0.5
[$Aib^{1,3}$,M]PTH(1-15)	174	±	12	0.3

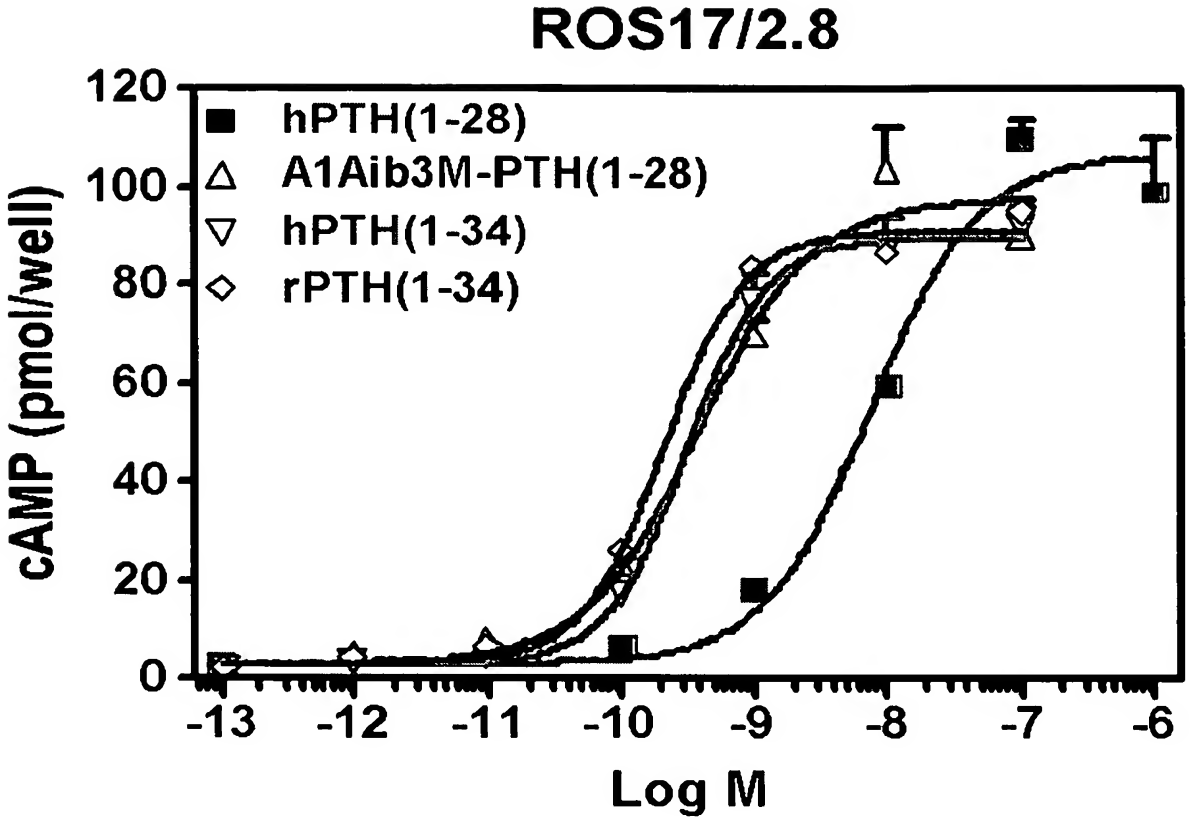
Figure 5B



Figures 6A-6D



Figures 7A and 7B



EC50 (nM)

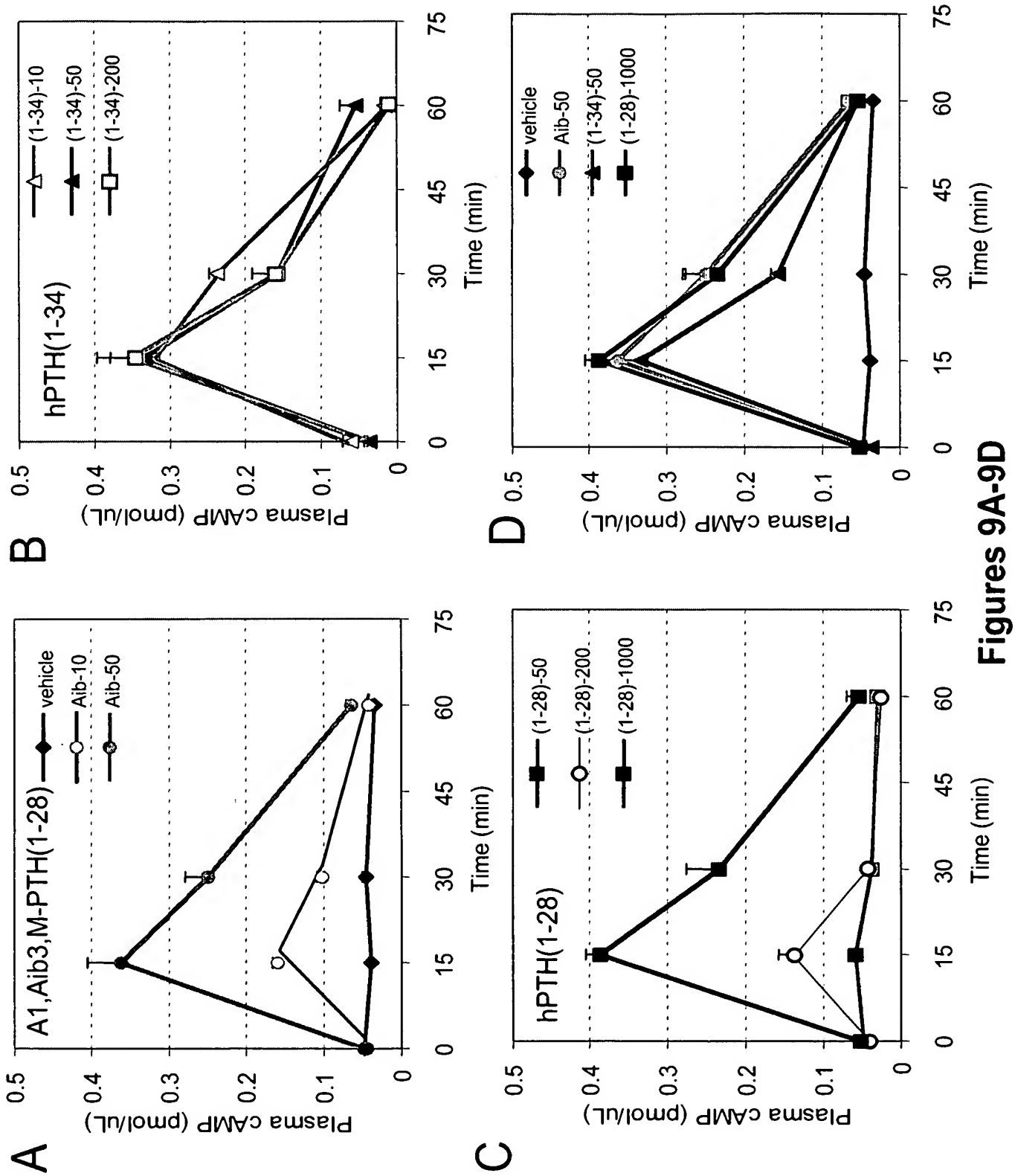
7.39 hPTH(1-28)NH2

0.37 $Ala^{1,12}, Aib^{3}, Gln^{10}, Har^{11}, Trp^{14}, Arg^{19}$-hPTH(1-28)$NH_2$

0.31 hPTH(1-34)NH2

0.21 rPTH(1-34)NH2

Figure 8



Figures 9A-9D

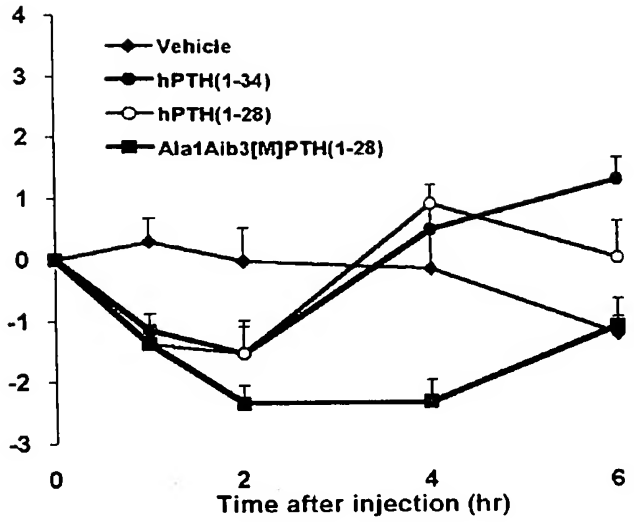


Figure 10A

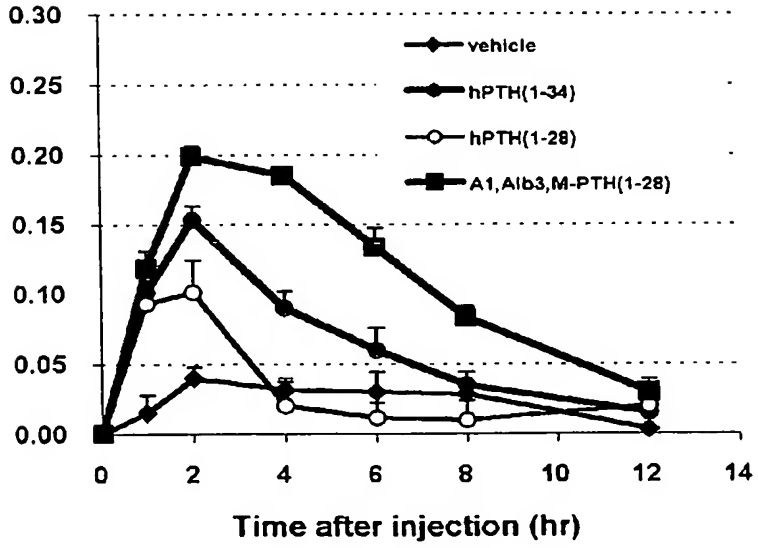


Figure 10B

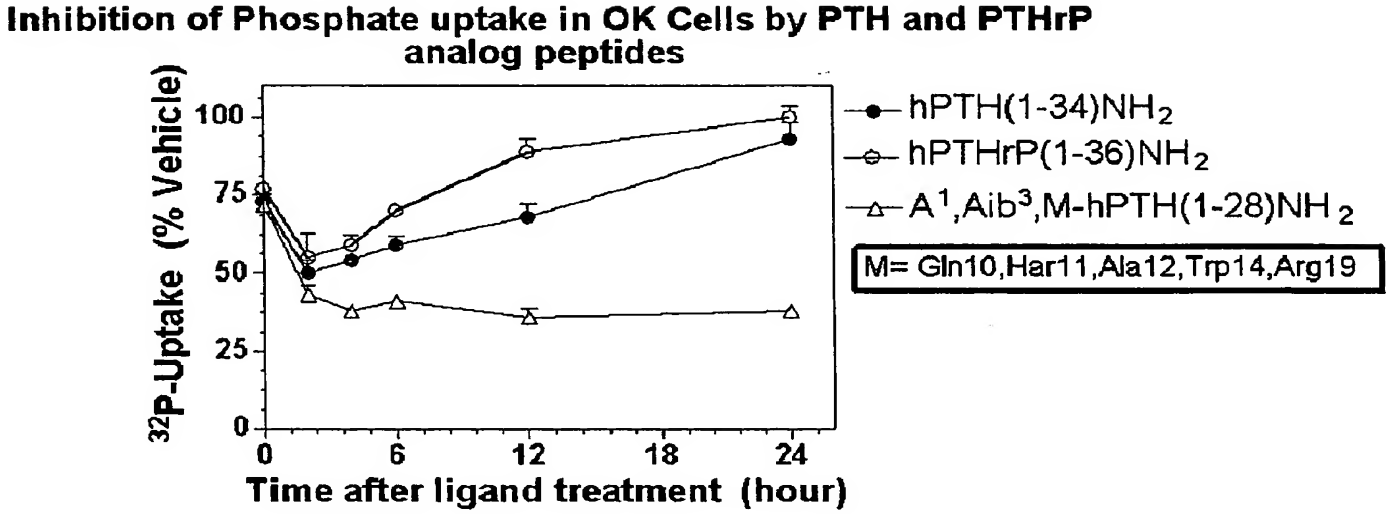

Inhibition of Phosphate uptake in OK Cells by PTH and PTHrP analog peptides
^{32}P-Uptake (% Vehicle)
100
75
50
25
0
0
6
12
18
24
Time after ligand treatment (hour)
hPTH(1-34)NH$_2$
hPTHrP(1-36)NH$_2$
A^1,Aib^3,M-hPTH(1-28)NH$_2$
M= Gln10,Har11,Ala12,Trp14,Arg19

Figure 11

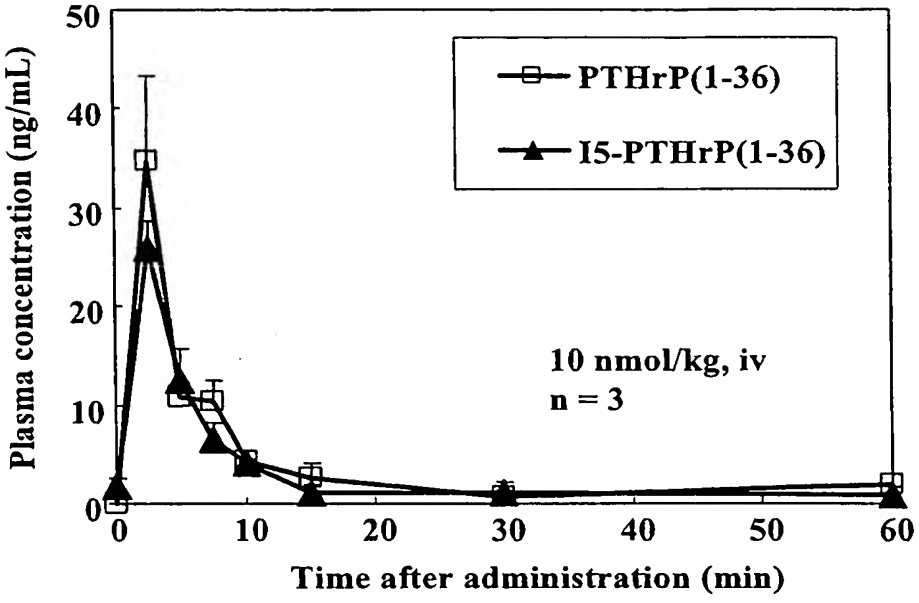
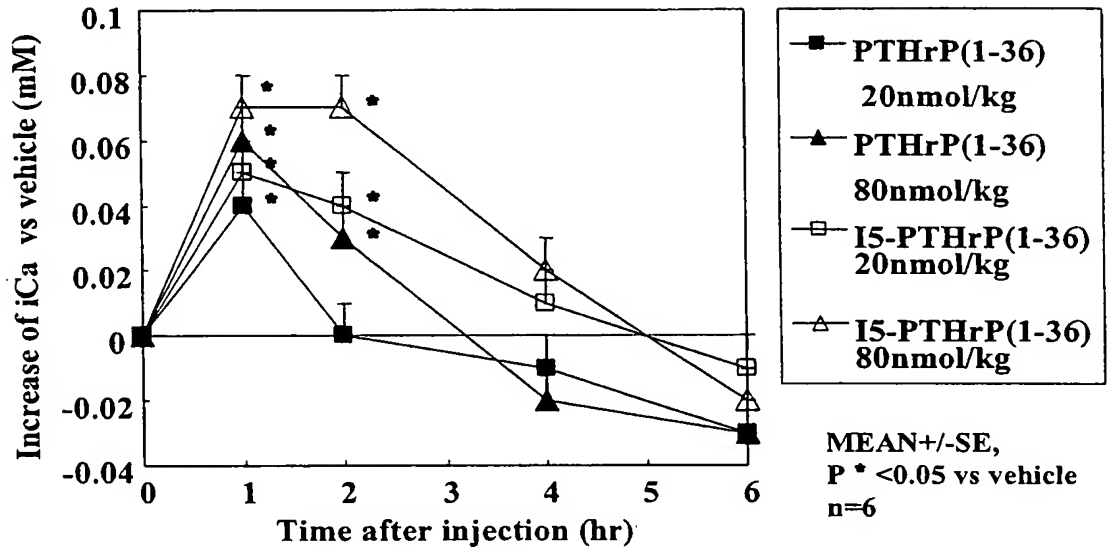

Plasma concentration (ng/mL)
50
40
30
20
10
0
0
10
20
30
40
50
60
Time after administration (min)
PTHrP(1-36)
I5-PTHrP(1-36)
10 nmol/kg, iv
n = 3

Figure 12

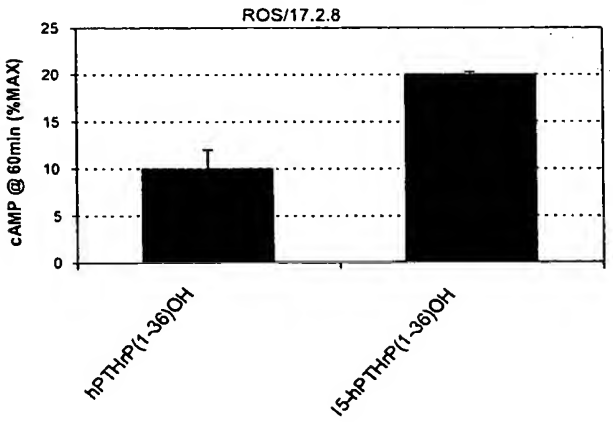
A



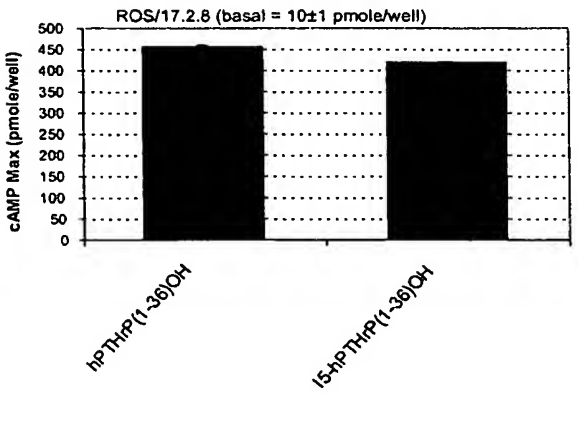
Binding to the R0 and RG forms of the rat PTHR *in vitro* **: PTHrP(1-36) and I5 substitution.**

MGH#		Binding IC_{50} nM Ro	Binding IC_{50} nM RG	n	selectivity ratio (R0:RG)
1207	hPTHrP(1-36)OH	20 ± 3	0.44 ± 0.07		46
1208	I5-hPTHrP(1-36)OH	2.3 ± 0.3	1.02 ± 0.03		2

B

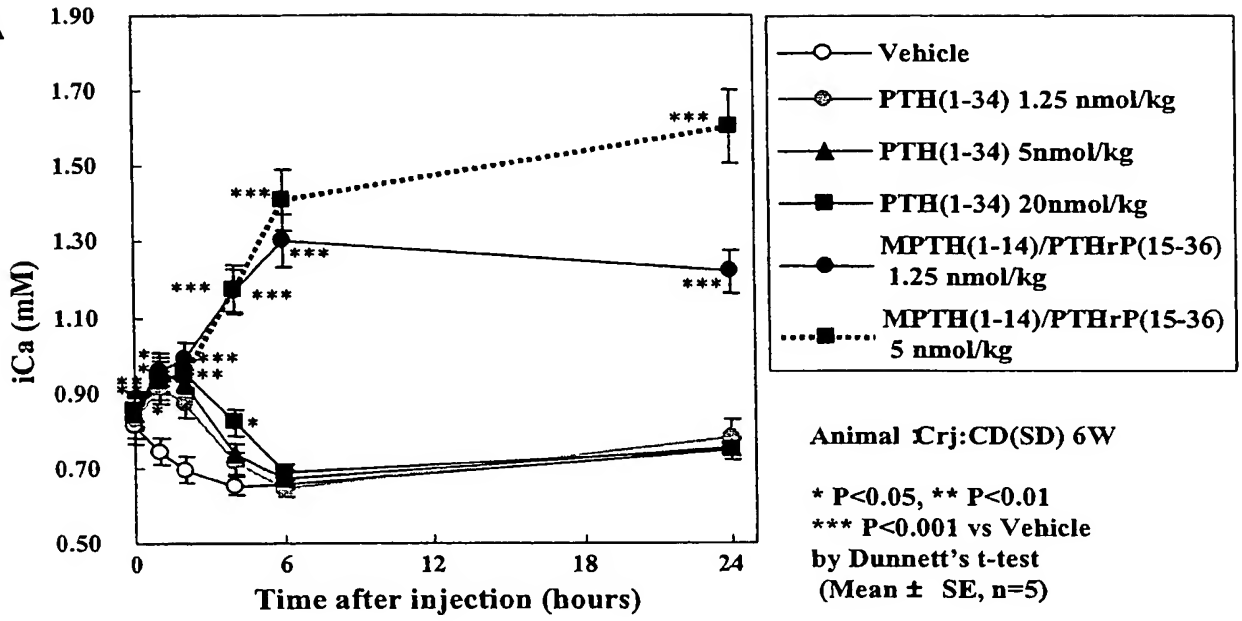


C



Figures 13A-13C

A

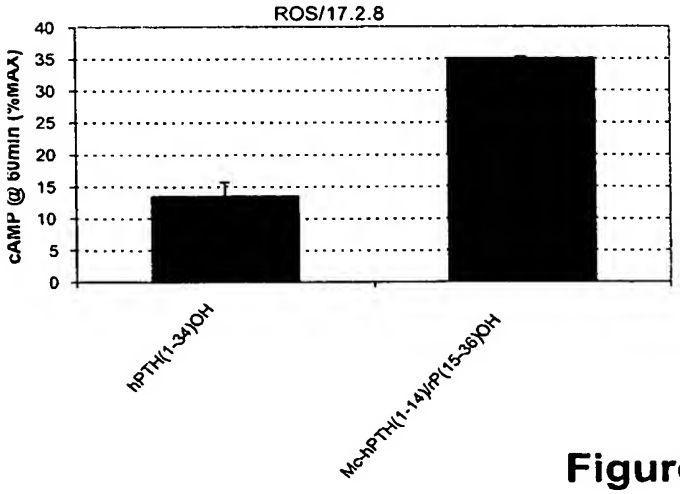


Binding to the R0 and RG forms of the rat PTHR *in vitro* **: PTH(1-34 and Mc-PTH(1-14)/PTHrP Hybrid.**

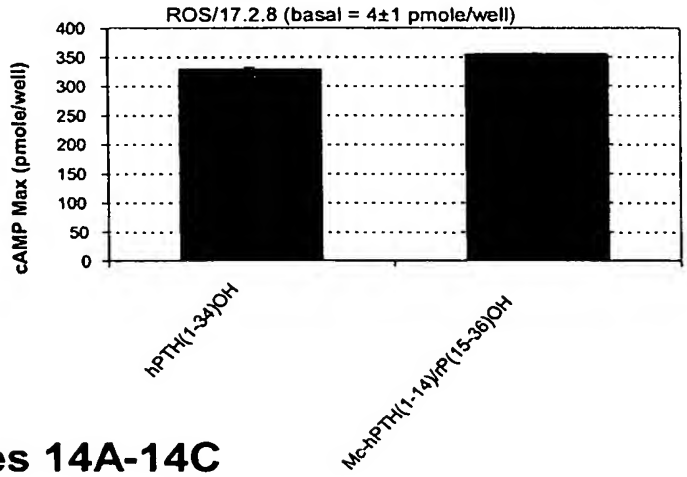
MGH#		Binding IC_{50} nM Ro	RG	n	selectivity ratio (R0:RG)
1202	hPTH(1-34)OH	93 ± 11	0.40 ± 0.03		233
1161	Mc-PTH(1-14)/PTHrP(15-36)OH	1.3 ± 0.3	0.81 ± 0.15	3	1.6

Mc= (coded amino acids only) A1,3,12,Q10,R11,W14,R19

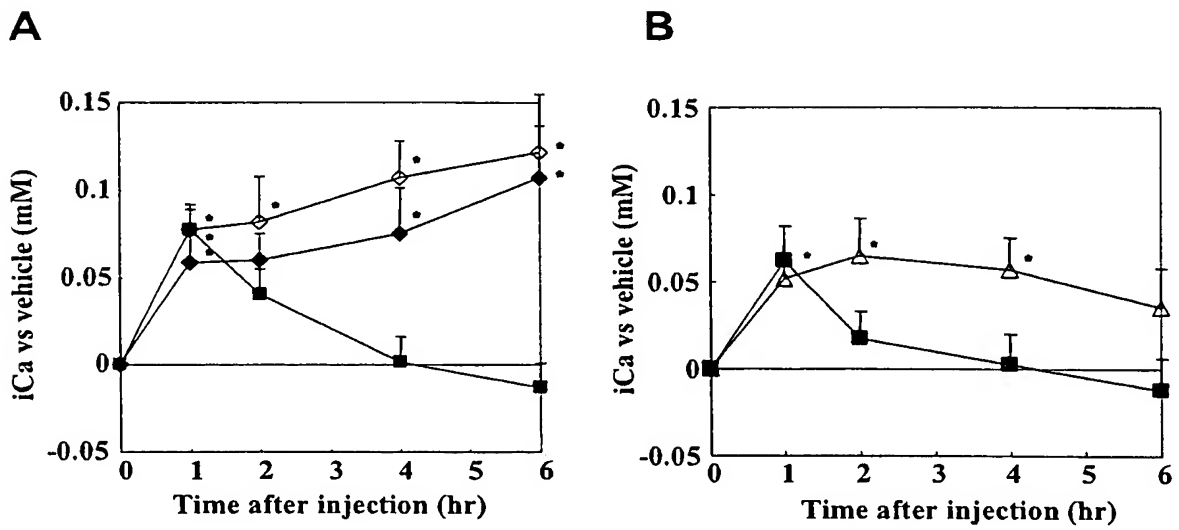
B



C



Figures 14A-14C



(PTH(1-34), M-PTH/PTHrP : 5nmol/kg, PTHrP : 20nmol/kg, iv, n = 6, MEAN+/-SE, P * <0.05 vs vehicle)

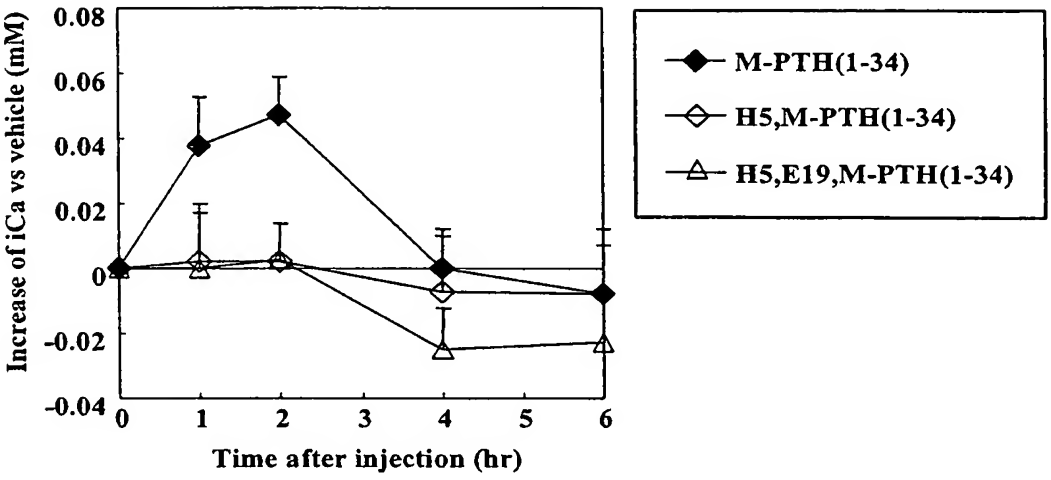
Binding to the R0 and RG forms of the rat PTHR *in vitro* **: McPTH(1-X)/(X-36) Hybrid peptides.**

MGH#		Binding IC_{50} nM R0		RG	n	selectivity ratio (R0:RG)
1202	hPTH(1-34)OH	93 ± 11		0.40 ± 0.03		233
1160	Mc-PTH(1-11)/PTHrP(12-36)OH	1.8 ± 0.7		0.73 ± 0.08	3	2.5
1161	Mc-PTH(1-14)/PTHrP(15-36)OH	1.3 ± 0.3		0.81 ± 0.15	3	1.6
1163	Mc-PTH(1-18)/PTHrP(19-36)OH	1.4 ± 0.4		0.82 ± 0.10	3	1.8

Mc= (coded amino acids only) A1,3,12,Q10,R11,W14,R19

Figures 15A and 15B

A



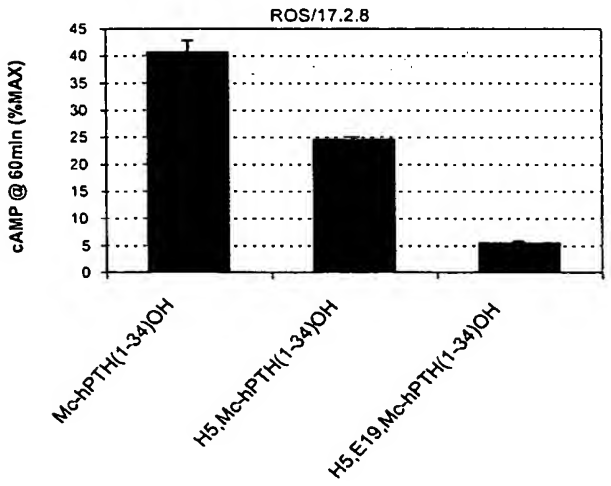
PTH(1-34), M-PTH34 analogs : 5nmol/kg, PTHrP : 20nmol/kg, iv, n = 6, MEAN+/-SE ,P * <0.05 vs vehicle

Binding to the R0 and RG forms of the rat PTHR *in vitro* : McPTH(1-34) and H5/E19 substitutions.

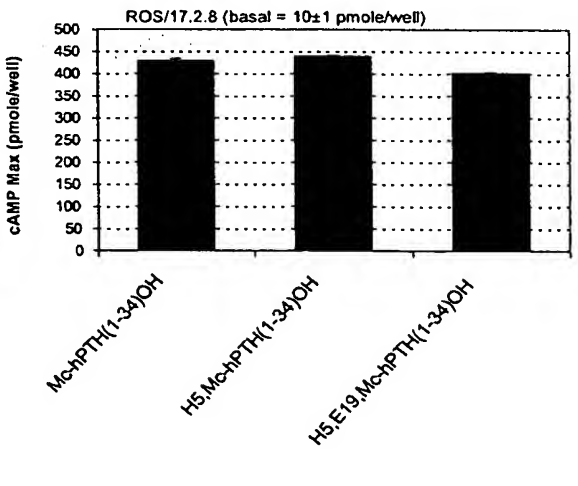
MGH#		Binding IC_{50} nM Ro	RG	n	selectivity ratio (R0:RG)
1205	Mc-hPTH(1-34)OH	1.9 ± 0.0	0.56 ± 0.06		3
1206	H5,Mc-hPTH(1-34)OH	26 ± 2	0.32 ± 0.02		81
1204	H5,E19,Mc-hPTH(1-34)OH	1389 ± 138	1.41 ± 0.26		984

Mc= (coded amino acids only) A1,3,12,Q10,R11,W14,R19

B

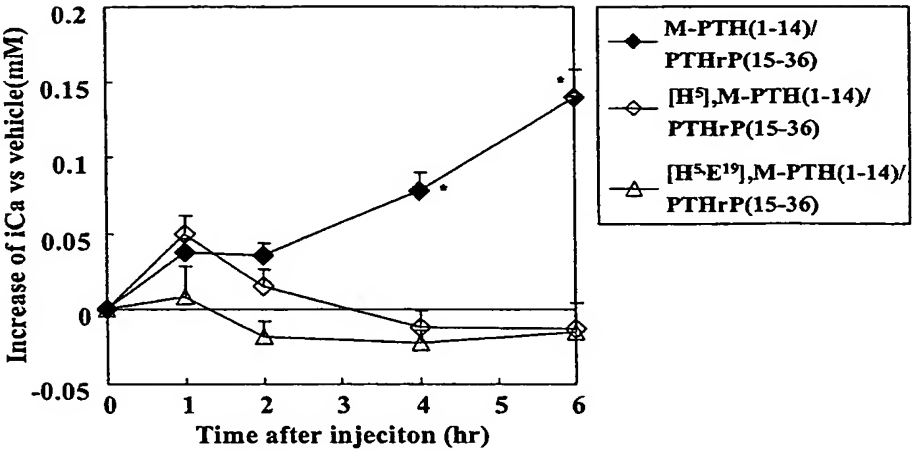


C



Figures 16A-16C

A



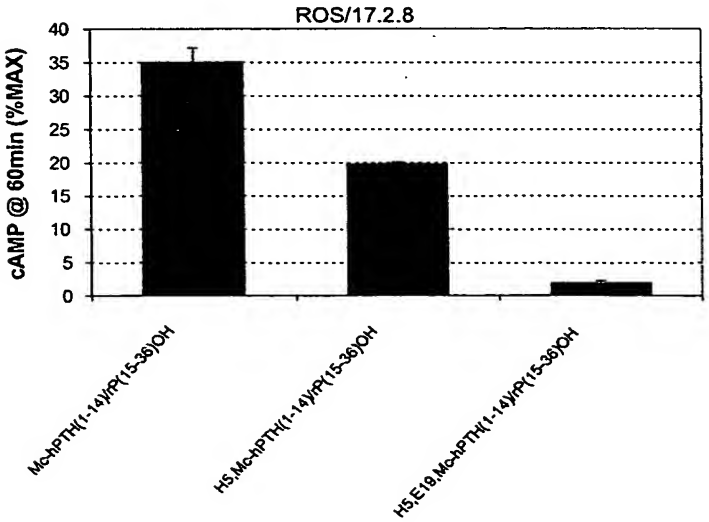
PTH(1-34), M-PTH34 : 5nmol/kg, PTHrP : 20nmol/kg, iv, n = 6, MEAN+/-SE ,P * <0.05 vs vehicle

Binding to the R0 and RG forms of the rat PTHR *in vitro* :
Mc-PTH/PTHrP Hybrids and H5/E19 substitutions.

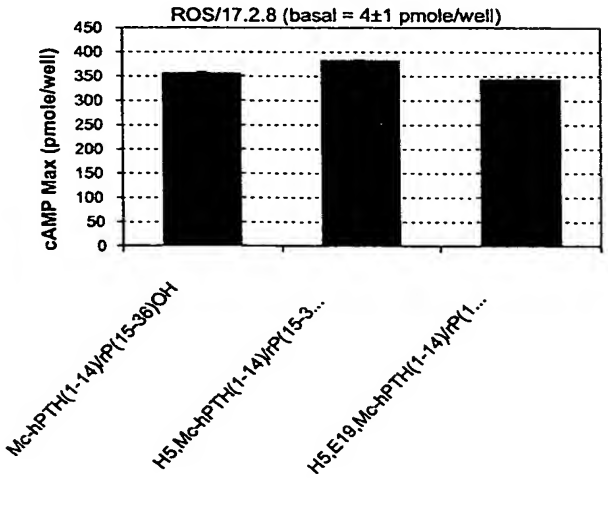
		Binding IC_{50} nM			selectivity
MGH#		Ro	RG	n	ratio (R0:RG)
1161	Mc-PTH(1-14)/PTHrP(15-36)OH	1.3 ± 0.3	0.81 ± 0.15	3	1.6
1212	H5,Mc-hPTH(1-14)/rP(15-36)OH	9.0 ± 2.5	1.7 ± 0.6		5.3
1214	H5,E19,Mc-hPTH(1-14)/rP(15-36)OH	197 ± 12.2	4.8 ± 2.1		41

Mc= (coded amino acids only) A1,3,12,Q10,R11,W14,R19

B

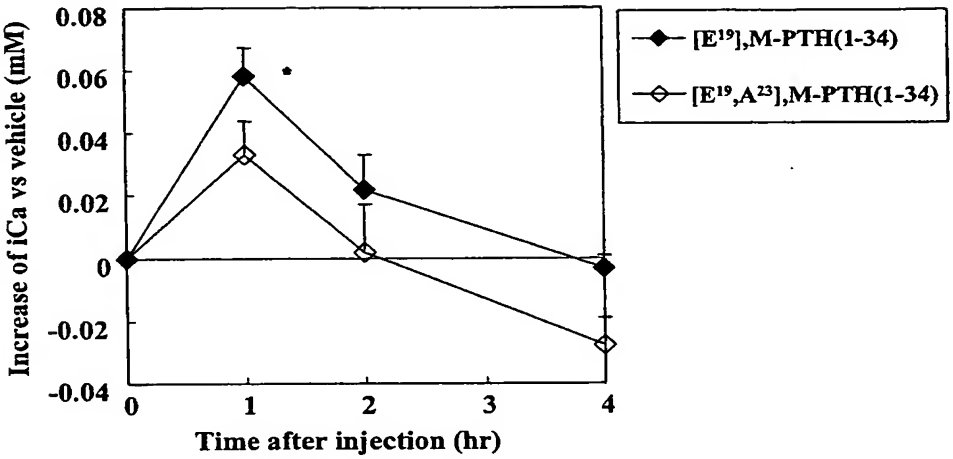


C



Figures 17A-17C

A



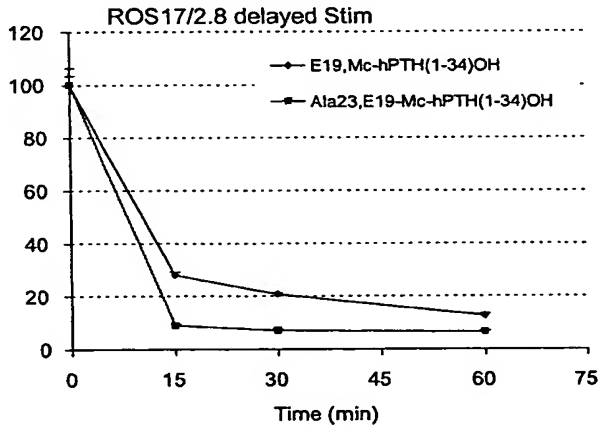
PTH(1-34), M-PTH34 : 5nmol/kg, PTHrP : 20nmol/kg, iv, n = 6,
MEAN+/-SE ,P * <0.05 vs vehicle

Binding to the R0 and RG forms of the rat PTHR *in vitro* **:**
E19-McPTH(1-34) and Ala23 substitution.

MGH#		Binding IC_{50} nM Ro		RG	n	selectivity ratio (R0:RG)
1203	E19,Mc-hPTH(1-34)OH	2.33 ± 0.19		0.57 ± 0.02		4.1
1312	Ala23,E19-Mc-hPTH(1-34)OH	20.3 ± 2.5		0.49 ± 0.05		41

Mc= (coded amino acids only) A1,3,12,Q10,R11,W14,R19

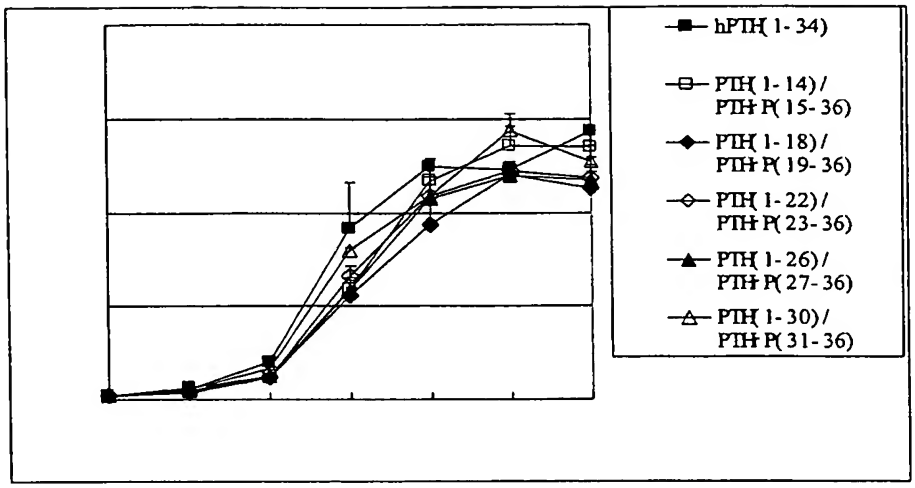
B



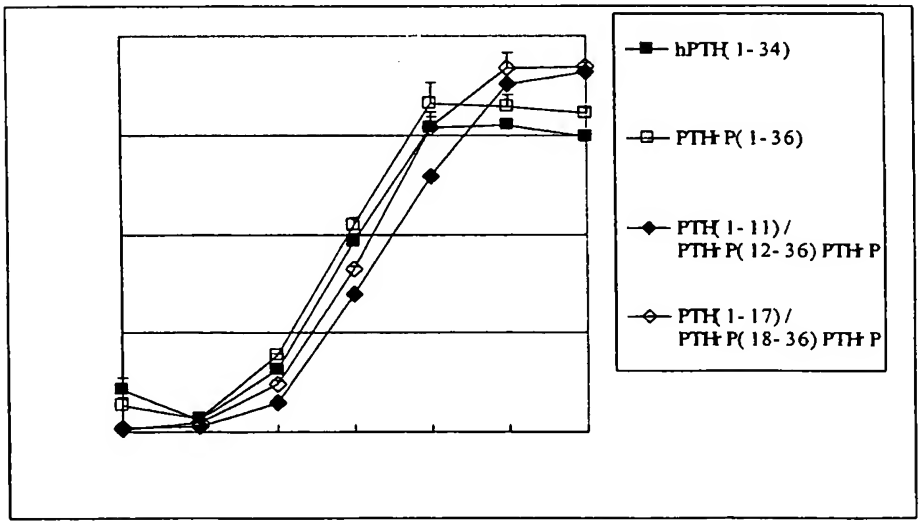
	Maximum cAMP (picomole/well)
Vehicle	0.5 ± 0.2
E19,Mc-hPTH(1-34)OH	51 ± 3
Ala23,E19-Mc-hPTH(1-34)OH	49 ± 2

Figures 18A and 18B

A

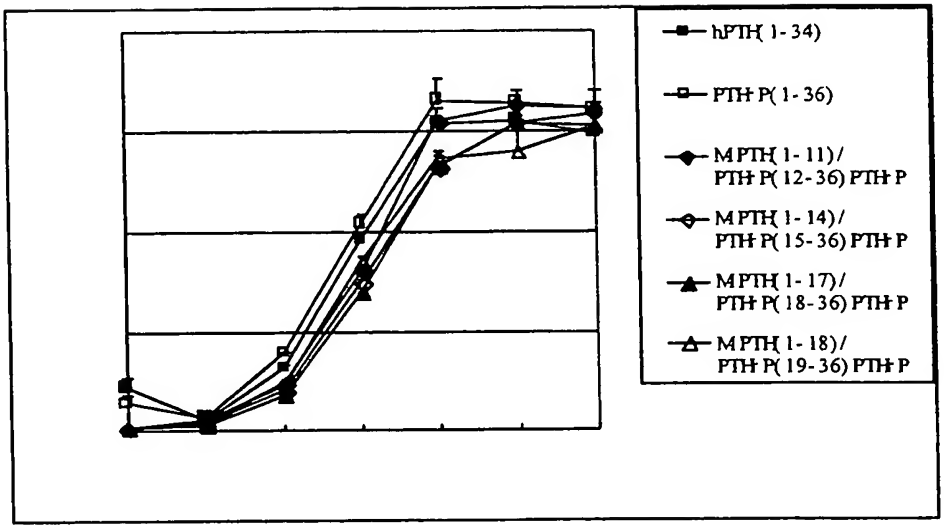


B

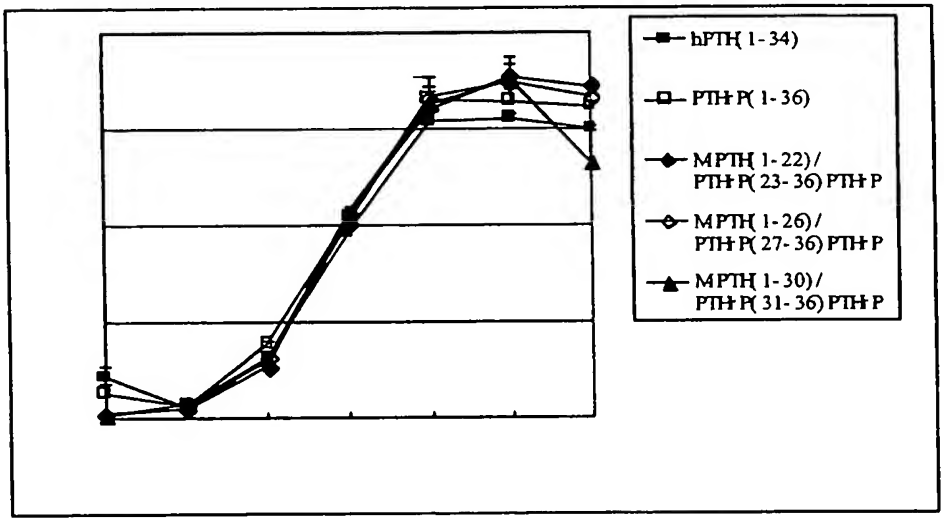


Figures 19A and 19B

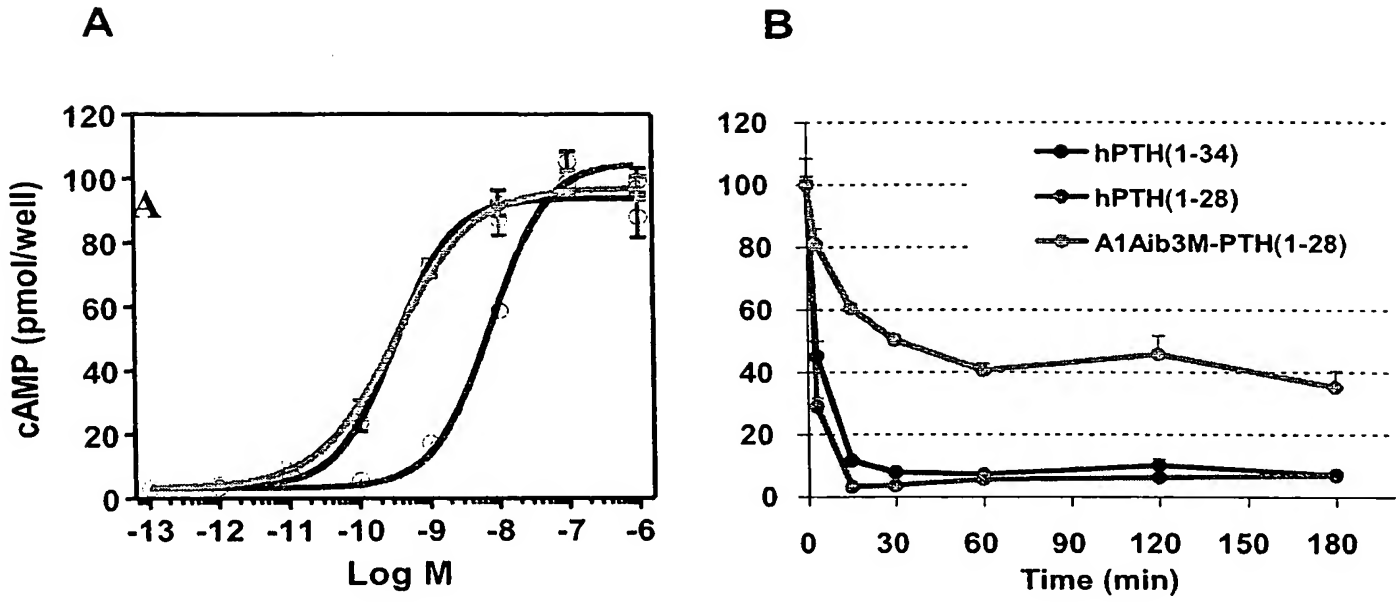
A



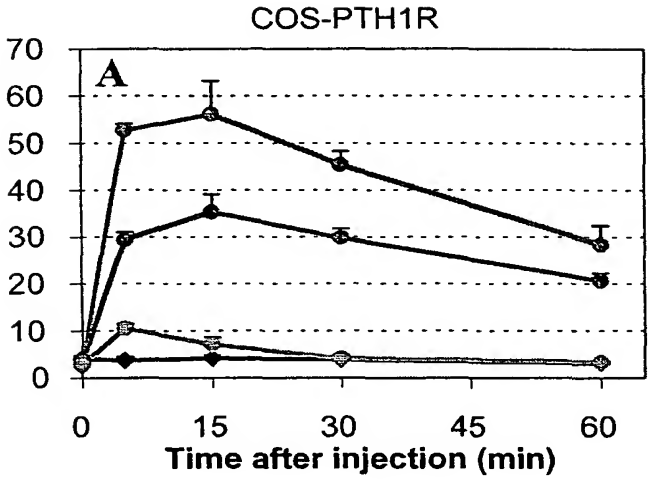
B

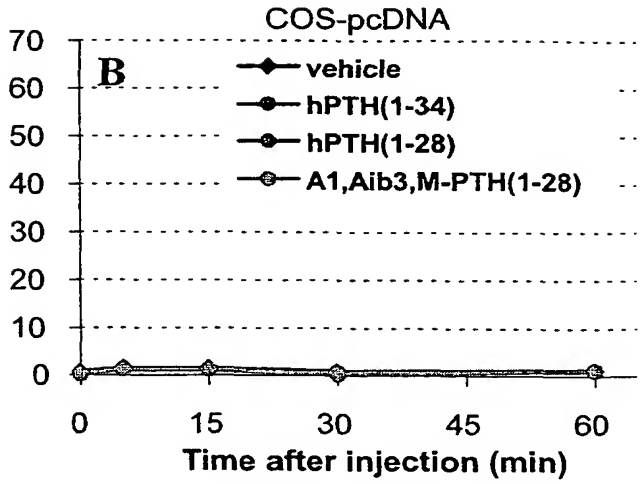


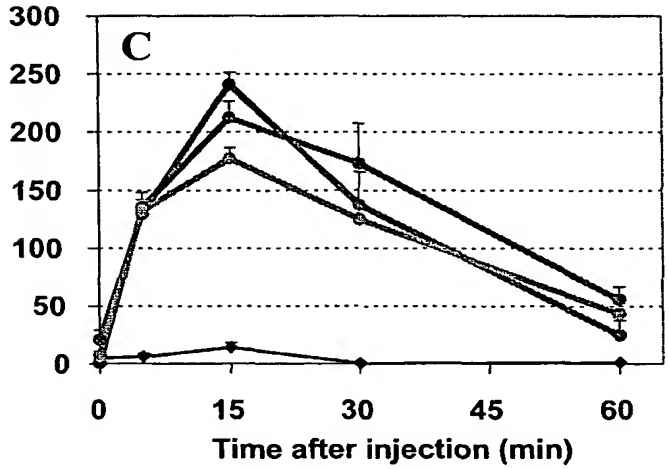
Figures 20A and 20B



Figures 21A and 21B







Figures 22A-22C

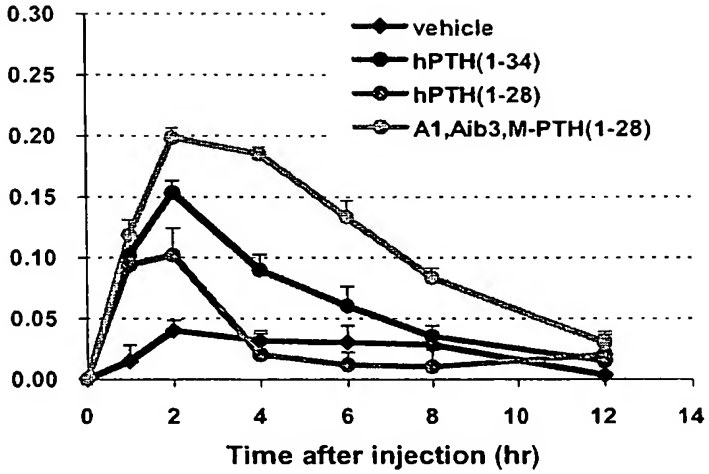

0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
Time after injection (hr)
vehicle
hPTH(1-34)
hPTH(1-28)
A1,Aib3,M-PTH(1-28)

Figure 23

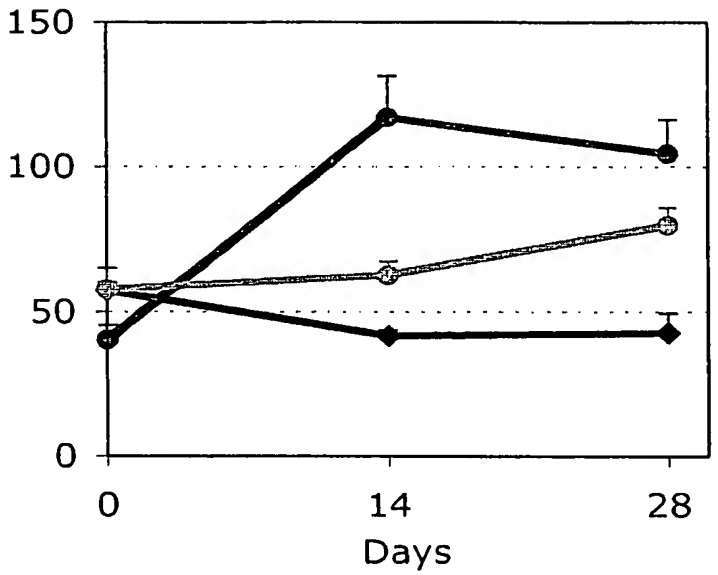


Figure 24A

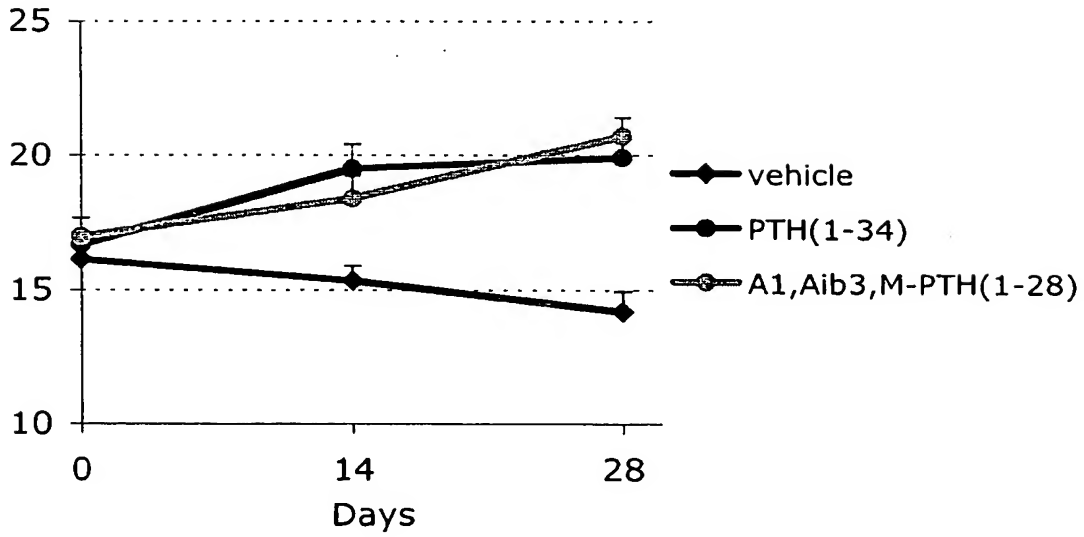


Figure 24B

cAMP signaling potency of PTH/PTHrP hybrid analogs on the human PTH receptor in HKRK-B7 cells

	cAMP in HRKRK-B7 cells EC50 (nM)
hPTH(1-34)	0.67
PTHrP(1-36)	0.55
PTH(1-14)/PTHrP(15-36)	1.35
PTH(1-18)/PTHrP(19-36)	1.34
PTH(1-22)/PTHrP(23-36)	0.89
PTH(1-26)/PTHrP(27-36)	1.07
PTH(1-30)/PTHrP(31-36)	0.78
M-PTH(1-11)/PTHrP(12-36)	0.98
M-PTH(1-14)/PTHrP(15-36)	1.25
M-PTH(1-17)/PTHrP(18-36)	1.28
M-PTH(1-18)/PTHrP(19-36)	0.74
M-PTH(1-22)/PTHrP(23-36)	0.66
M-PTH(1-26)/PTHrP(27-36)	0.66
M-PTH(1-30)/PTHrP(31-36)	0.60

Figure 25

Competition analysis of R0 and RG binding of PTH/PTHrP analogs with the human PTH receptor

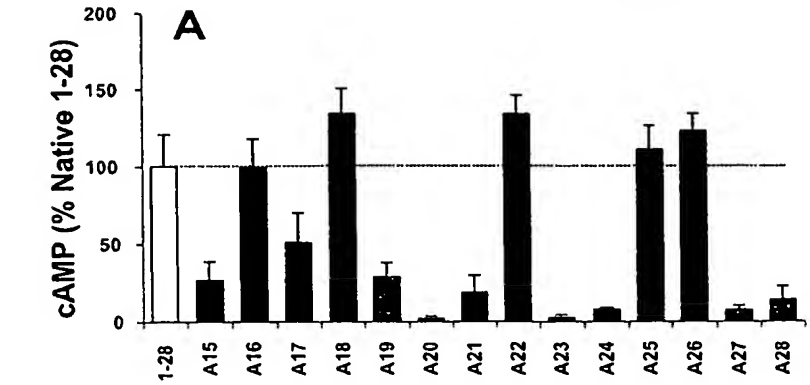
MGH #	peptide	R0 memb: hPTH1R*PTH(1-34)+GTPgS			RG memb: hPTH1R+dnGS *M-PTH(1-15)			R0/RG selectivity	Fold vs hPTH(1-34)	Group
1177	rPTH(1-34)	2.4	±	0.1	0.24	±	0.00	10	0.2	R0
1202	hPTH(1-34)	8.7	±	1.2	0.13	±	0.02	67	1.0	
1207	hPTHrP(1-36)	37.7	±	4.7	0.14	±	0.02	260	3.9	RG
1208	I5-hPTHrP(1-36)	3.3	±	0.8	0.38	±	0.04	9	0.1	R0
1203	E19,Mc-hPTH(1-34)	2.5	±	1.1	0.22	±	0.01	11	0.2	R0
1204	H5,E19,Mc-hPTH(1-34)	67.1	±	19.5	0.34	±	0.05	199	3.0	RG
1205	Mc-hPTH(1-34) (R19)	1.7	±	0.8	0.26	±	0.03	7	0.1	R0
1206	H5,M-hPTH(1-34) (R19)	3.2	±	1.4	0.09	±	0.02	35	0.5	R0
1209	H5-hPTH(1-14)/rP(15-36)	5.0	±	0.6	0.75	±	0.27	7	0.1	R0
1210	E19-hPTH(1-14)/rP(15-36)	7.9	±	0.4	0.98	±	0.35	8	0.1	R0
1211	H5,E19-hPTH(1-14)/rP(15-36)	12.9	±	1.3	0.57	±	0.17	23	0.3	RG
1212	H5,Mc-hPTH(1-14)/rP(15-36)	20.4	±	2.7	0.53	±	0.16	38	0.6	RG
1213	E19,Mc-hPTH(1-14)/rP(15-36)	11.4	±	0.6	0.84	±	0.26	14	0.2	RG
1214	H5,E19,Mc-hPTH(1-14)/rP(15-36)	321.1	±	24.0	0.65	±	0.15	496	7.4	RG
1155	PTH(1-11)/PTHrP(12-36)	2.8	±	0.9	0.18	±	0.04	16	0.2	R0
1156	PTH(1-17)/PTHrP(18-36)	4.8	±	0.8	0.23	±	0.03	22	0.3	R0
1157	PTH(1-22)/PTHrP(23-36)	16.8	±	2.9	0.22	±	0.12	76	1.1	RG
1158	PTH(1-26)/PTHrP(27-36)	7.9	±	0.8	0.31	±	0.10	26	0.4	R0
1159	PTH(1-30)/PTHrP(31-36)	6.6	±	1.9	0.17	±	0.04	39	0.6	R0
1160	Mc-PTH(1-11)/PTHrP(12-36)	2.1	±	0.5	0.56	±	0.09	4	0.1	R0
1161	Mc-PTH(1-14)/PTHrP(15-36)	2.7	±	1.0	0.67	±	0.05	4	0.1	R0
1162	Mc-PTH(1-17)/PTHrP(18-36)	1.9	±	0.4	0.23	±	0.02	8	0.1	R0
1163	Mc-PTH(1-18)/PTHrP(19-36)	1.7	±	0.2	0.13	±	0.02	13	0.2	R0
1164	E19,Mc-PTH(1-22)/PTHrP(23-36)	7.8	±	2.6	0.66	±	0.02	12	0.2	R0
1165	E19,Mc-PTH(1-26)/PTHrP(27-36)	2.6	±	0.9	0.15	±	0.04	18	0.3	R0
1166	E19,Mc-PTH(1-30)/PTHrP(31-36)	3.0	±	0.5	0.21	±	0.03	14	0.2	R0
1311	A20,E19,Mc-PTH(1-34)OH	530.0	±	81.4	0.69	±	0.15	764	11.4	RG
1312	A23,E19,Mc-PTH(1-34)OH	12.5	±	2.0	0.14	±	0.04	87	1.3	RG
1313	A24,E19,Mc-PTH(1-34)OH	64.0	±	10.4	0.23	±	0.08	278	4.1	RG
1314	A23,M-PTH(1-34)OH	4.5	±	1.4	0.21	±	0.14	22	0.3	R0
1347	A20-M-PTH(1-34)OH	31.9	±	10.5	0.40	±	0.09	80	1.2	RG
1348	F23-M-PTH(1-34)OH	1.2	±	0.4	0.23	±	0.07	5	0.1	R0
809	A1,Aib3,M-PTH(1-28)NH2	1.7	±	0.3	0.5	±	0.07	3.4	0.1	R0
	Mc=A1,3,12,Q10,R11,W14,R19									
	Cter: OH (free carboxy) unless otherwise noted									

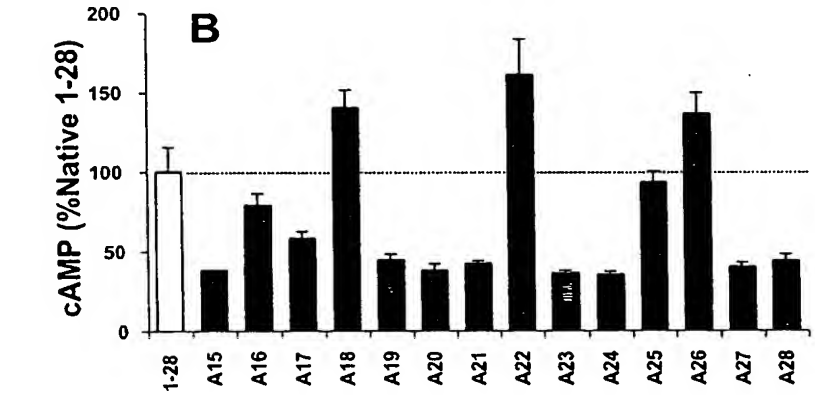
Figure 26A

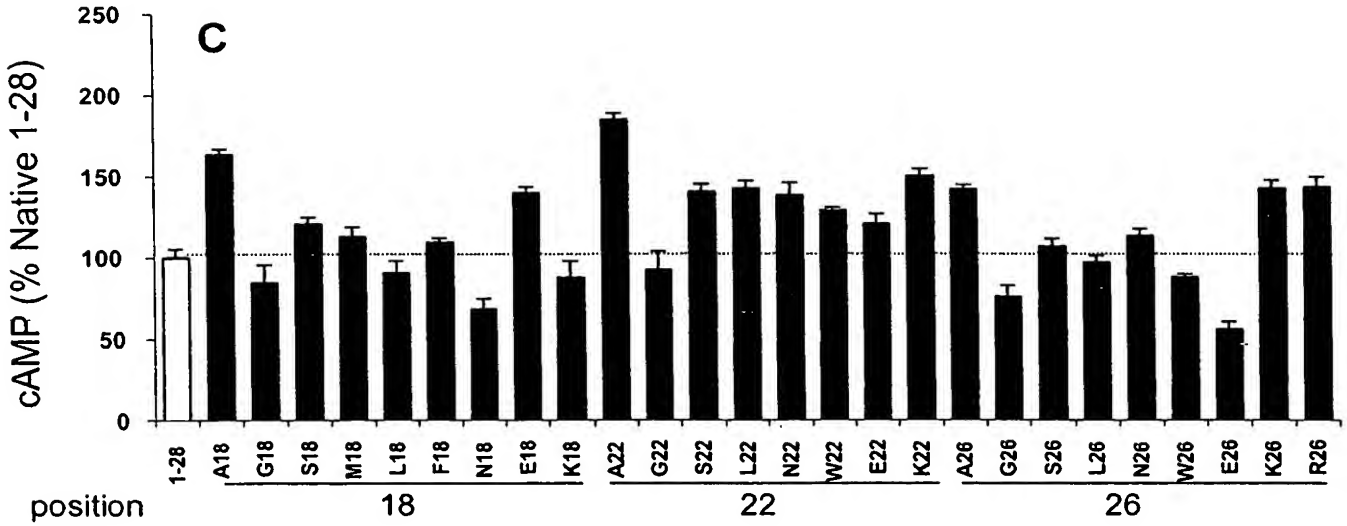
Competition analysis of R0 and RG binding of PTH/PTHrP analogs with the human PTH receptor

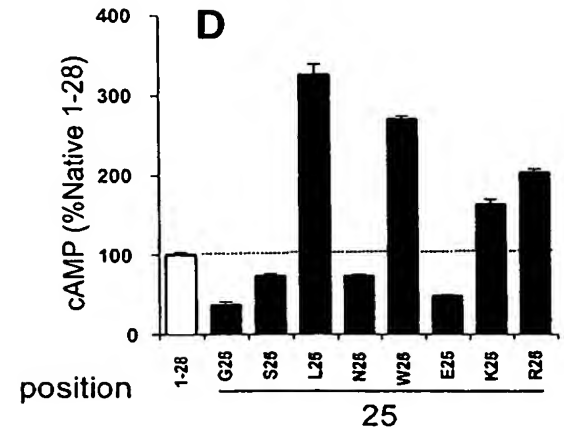
MGH #	peptide	R0 memb: hPTH1R*PTH(1-34)+GTPgS			RG memb: hPTH1R+dnGS *M-PTH(1-15)			R0/RG selectivity	Fold vs hPTH(1-34)	Group
1348	F23-M-PTH(1-34)OH	1.2	±	0.4	0.23	±	0.07	5	0.4	R0
1163	Mc-PTH(1-18)/PTHrP(19-36)	1.7	±	0.2	0.13	±	0.02	13	1.0	R0
809	A1,Aib3,M-PTH(1-28)NH2	1.7	±	0.3	0.5	±	0.07	3.4	0.3	R0
1205	Mc-hPTH(1-34) (R19)	1.7	±	0.8	0.26	±	0.03	7	0.5	R0
1162	Mc-PTH(1-17)/PTHrP(18-36)	1.9	±	0.4	0.23	±	0.02	8	0.6	R0
1160	Mc-PTH(1-11)/PTHrP(12-36)	2.1	±	0.5	0.56	±	0.09	4	0.3	R0
1177	rPTH(1-34)	2.4	±	0.1	0.24	±	0.00	10	0.8	R0
1203	E19,Mc-hPTH(1-34)	2.5	±	1.1	0.22	±	0.01	11	0.9	R0
1165	E19,Mc-PTH(1-26)/PTHrP(27-36)	2.6	±	0.9	0.15	±	0.04	18	1.3	R0
1161	Mc-PTH(1-14)/PTHrP(15-36)	2.7	±	1.0	0.67	±	0.05	4	0.3	R0
1155	PTH(1-11)/PTHrP(12-36)	2.8	±	0.9	0.18	±	0.04	16	1.3	R0
1166	E19,Mc-PTH(1-30)/PTHrP(31-36)	3.0	±	0.5	0.21	±	0.03	14	1.1	R0
1206	H5,M-hPTH(1-34) (R19)	3.2	±	1.4	0.09	±	0.02	35	2.7	R0
1208	I5-hPTHrP(1-36)	3.3	±	0.8	0.38	±	0.04	9	0.7	R0
1314	A23,M-PTH(1-34)OH	4.5	±	1.4	0.21	±	0.14	22	1.7	R0
1156	PTH(1-17)/PTHrP(18-36)	4.8	±	0.8	0.23	±	0.03	22	1.7	R0
1209	H5-hPTH(1-14)/rP(15-36)	5.0	±	0.6	0.75	±	0.27	7	0.5	R0
1159	PTH(1-30)/PTHrP(31-36)	6.6	±	1.9	0.17	±	0.04	39	3.0	R0
1164	E19,Mc-PTH(1-22)/PTHrP(23-36)	7.8	±	2.6	0.66	±	0.02	12	0.9	R0
1210	E19-hPTH(1-14)/rP(15-36)	7.9	±	0.4	0.98	±	0.35	8	0.6	R0
1158	PTH(1-26)/PTHrP(27-36)	7.9	±	0.8	0.31	±	0.10	26	2.0	R0
1202	hPTH(1-34)	8.7	±	1.2	0.13	±	0.02	67	5.2	
1213	E19,Mc-hPTH(1-14)/rP(15-36)	11.4	±	0.6	0.84	±	0.26	14	1.0	RG
1312	A23,E19,Mc-PTH(1-34)OH	12.5	±	2.0	0.14	±	0.04	87	6.7	RG
1211	H5,E19-hPTH(1-14)/rP(15-36)	12.9	±	1.3	0.57	±	0.17	23	1.7	RG
1157	PTH(1-22)/PTHrP(23-36)	16.8	±	2.9	0.22	±	0.12	76	5.8	RG
1212	H5,Mc-hPTH(1-14)/rP(15-36)	20.4	±	2.7	0.53	±	0.16	38	3.0	RG
1347	A20-M-PTH(1-34)OH	31.9	±	10.5	0.40	±	0.09	80	6.2	RG
1207	hPTHrP(1-36)	37.7	±	4.7	0.14	±	0.02	260	20.1	RG
1313	A24,E19,Mc-PTH(1-34)OH	64.0	±	10.4	0.23	±	0.08	278	21.4	RG
1204	H5,E19,Mc-hPTH(1-34)	67.1	±	19.5	0.34	±	0.05	199	15.3	RG
1214	H5,E19,Mc-hPTH(1-14)/rP(15-36)	321.1	±	24.0	0.65	±	0.15	496	38.2	RG
1311	A20,E19,Mc-PTH(1-34)OH	530.0	±	81.4	0.69	±	0.15	764	58.9	RG
	Mc=A1,3,12,Q10,R11,W14,R19									
	Cter: OH (free carboxy) unless noted otherwise									

Figure 26B

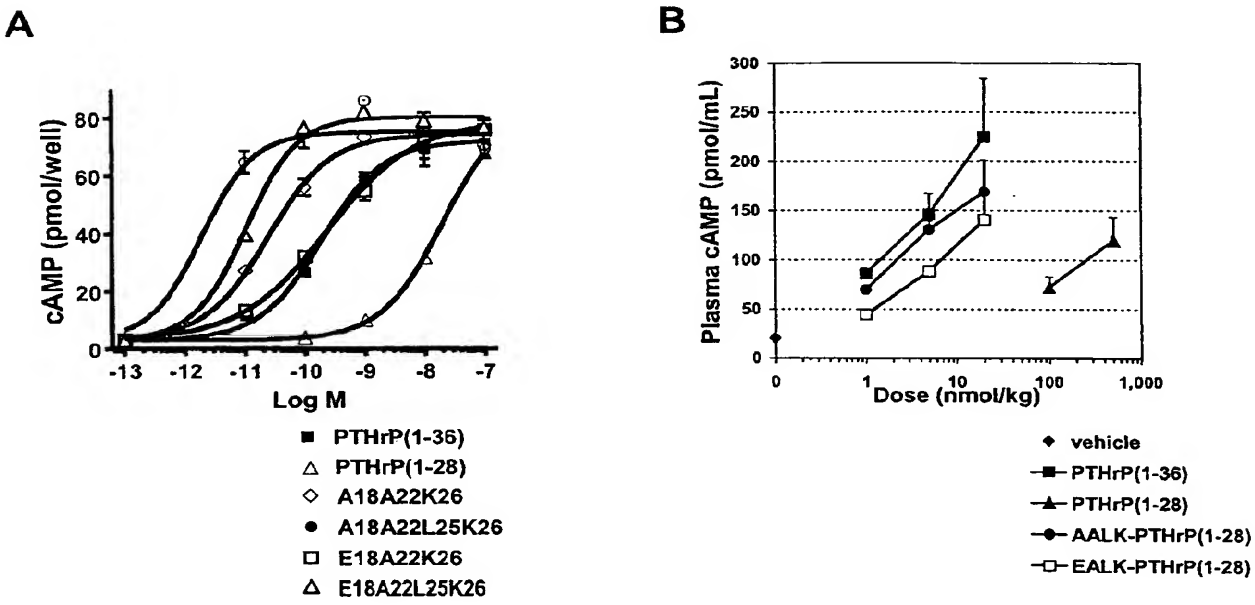




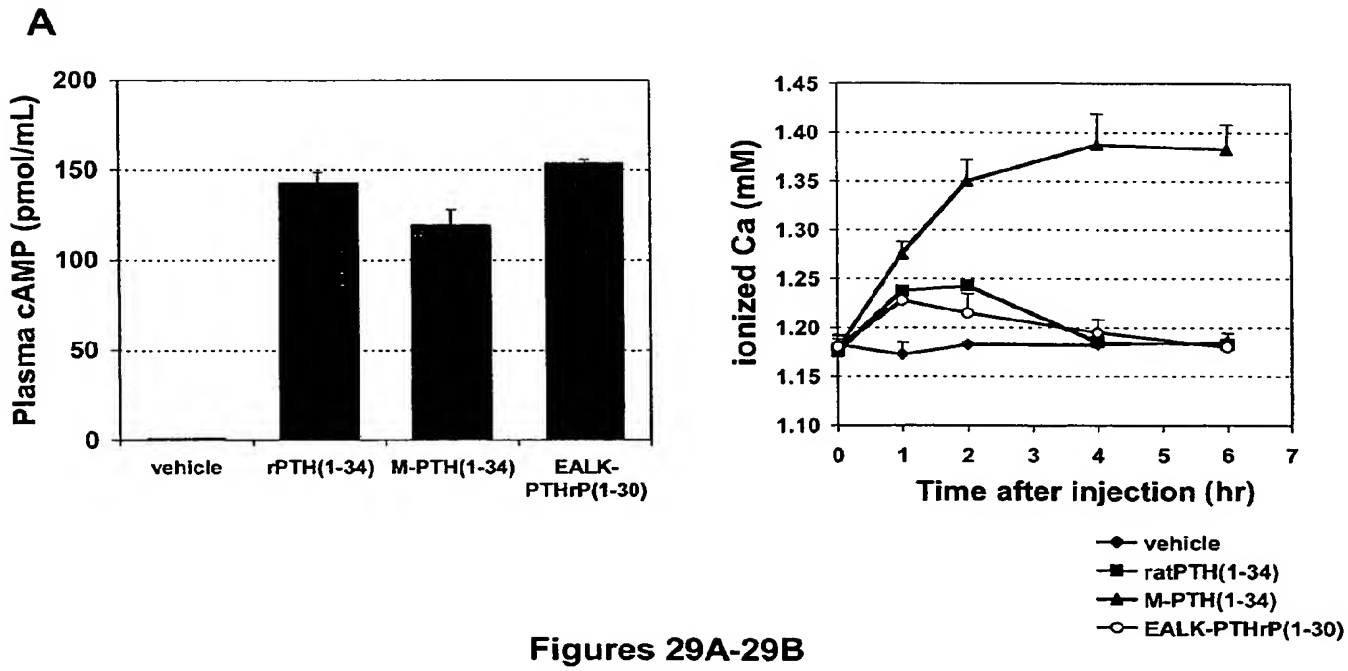




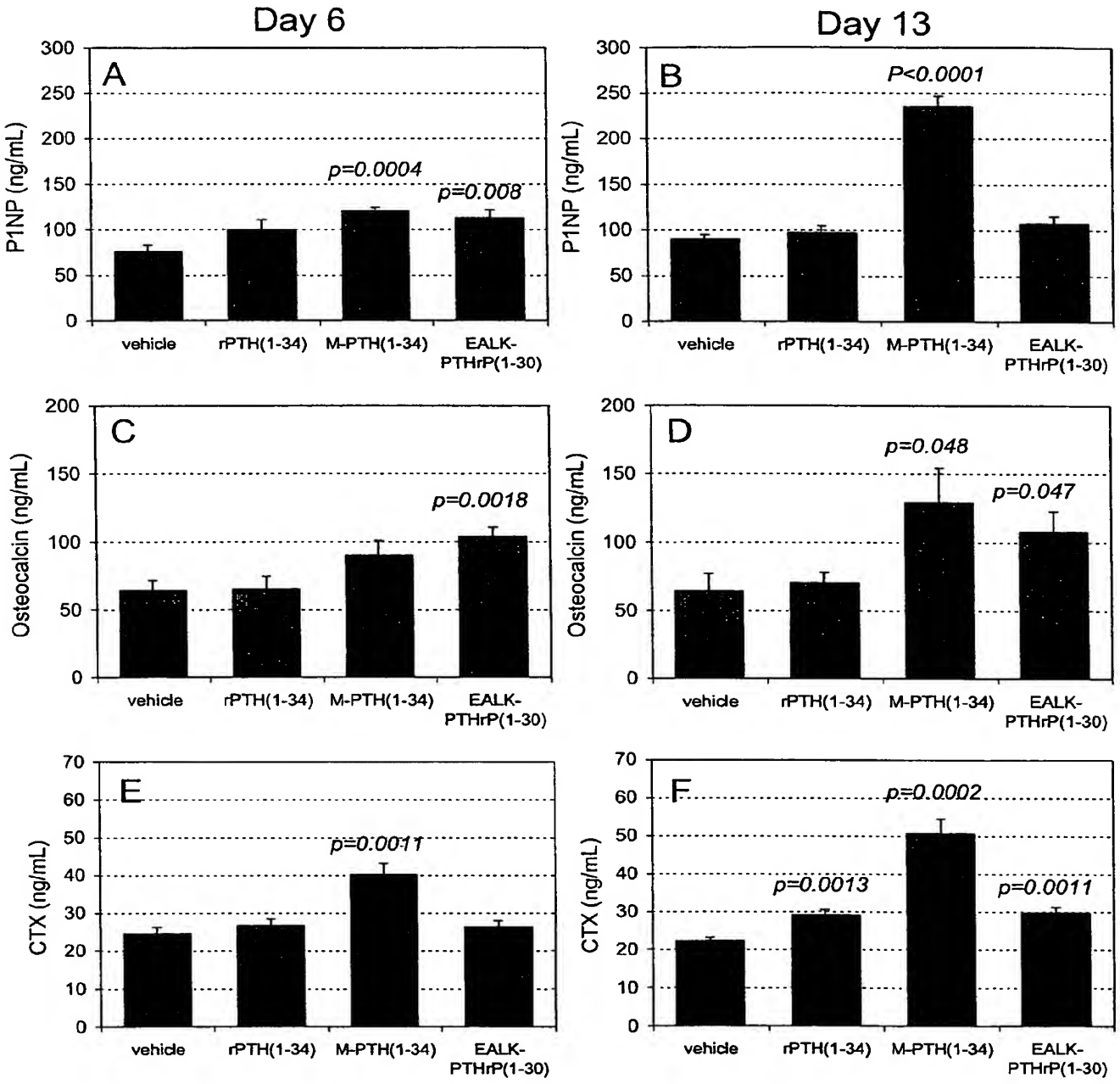
Figures 27A-27D



Figures 28A-28B



Figures 29A-29B



Figures 30A-30F

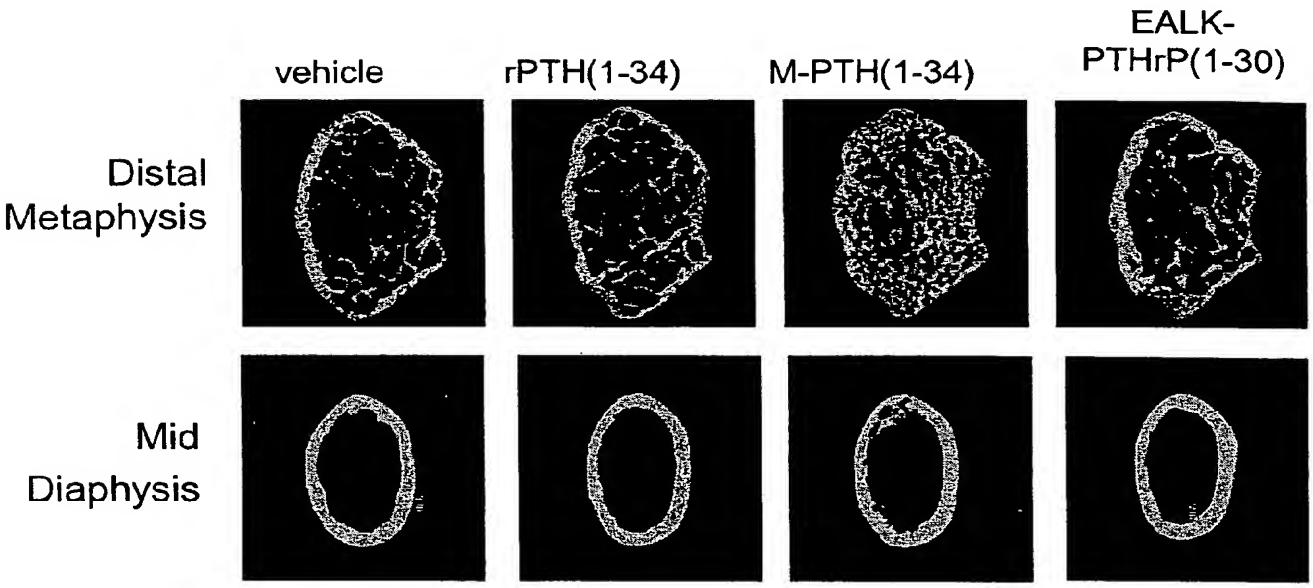
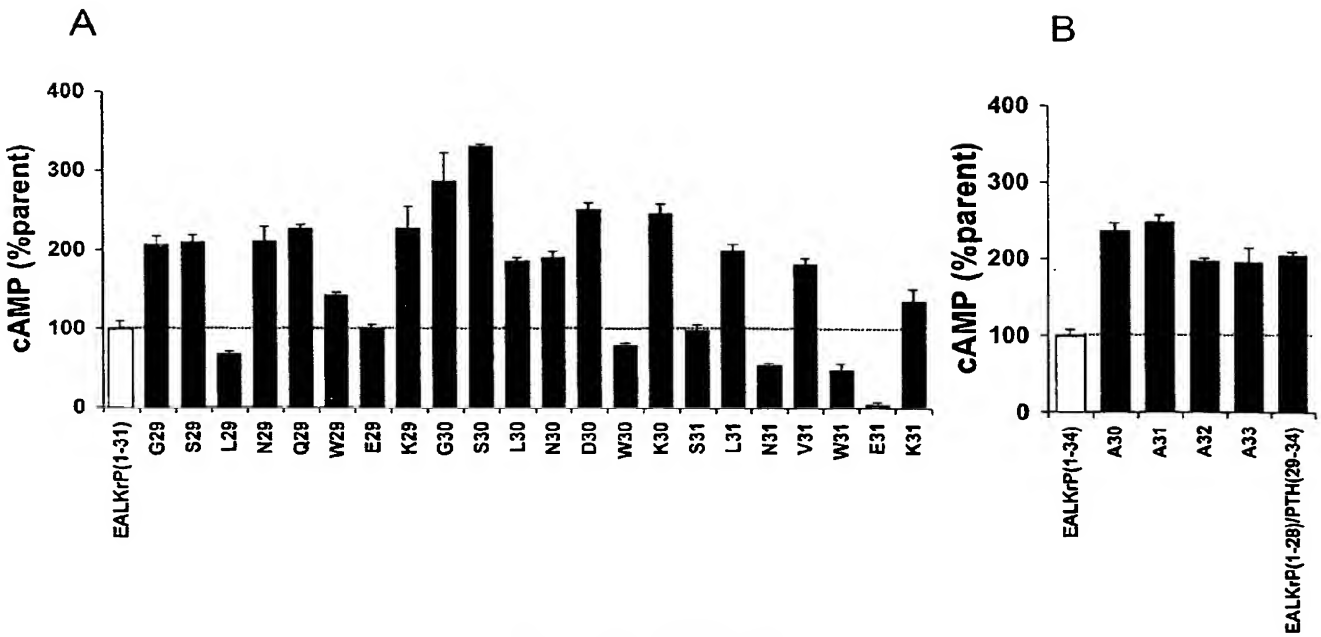
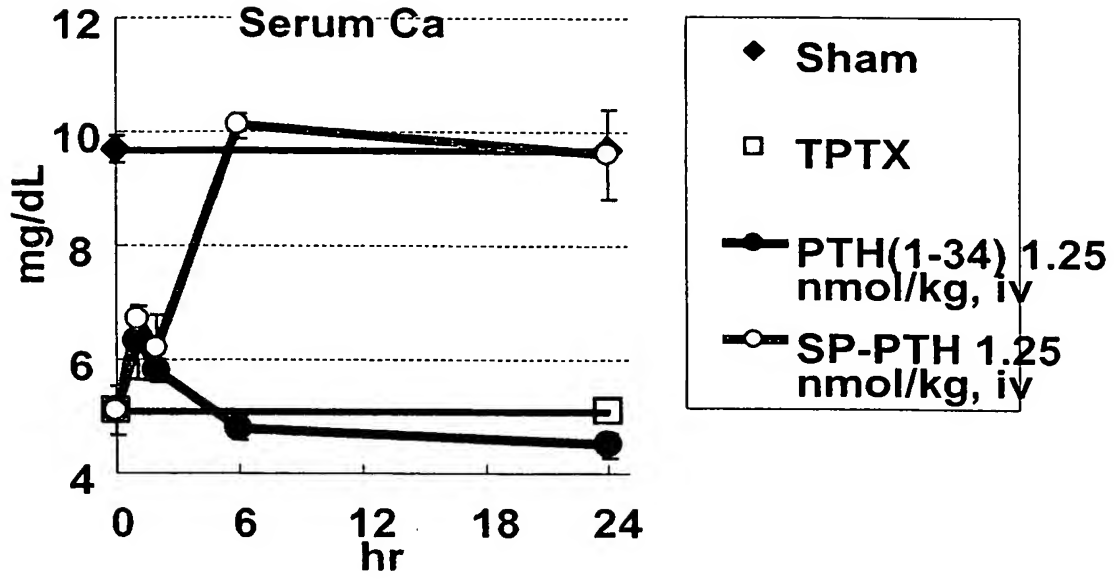


Figure 31

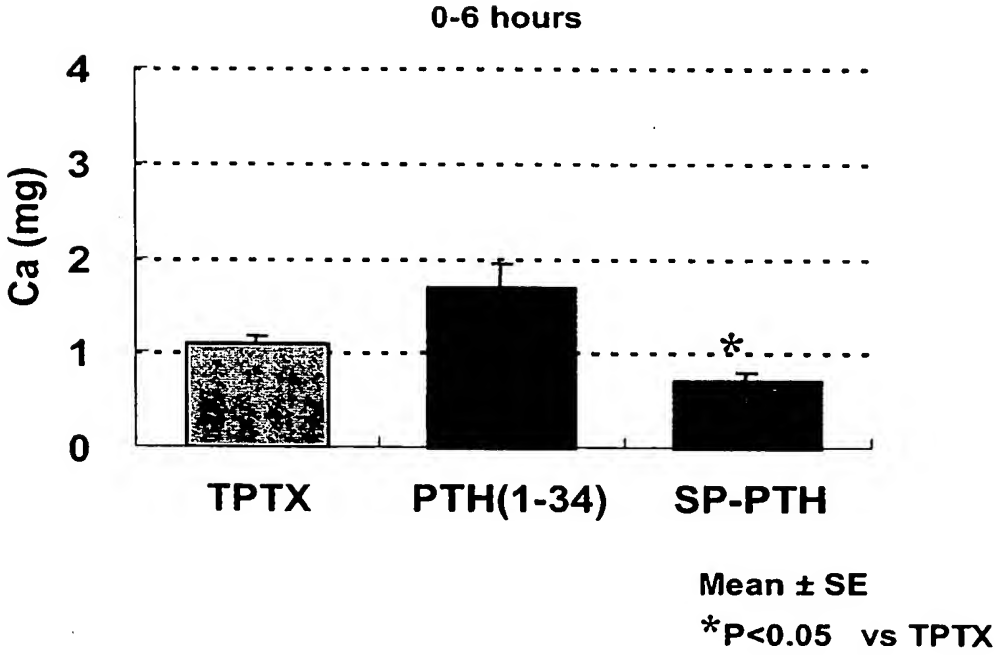


Figures 32A-32B



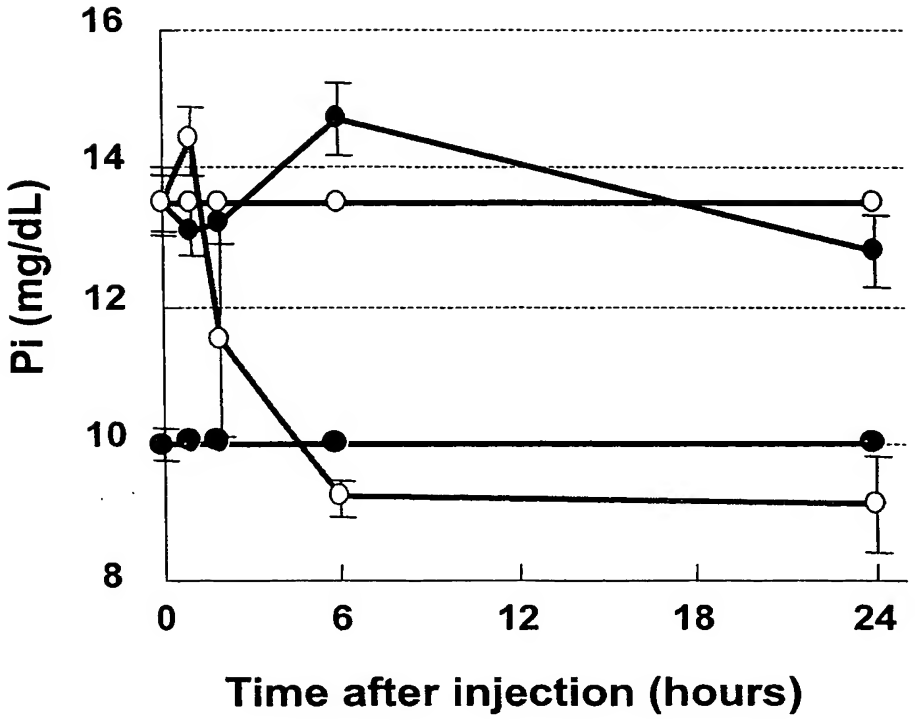
Animal : Crj:CD(SD) rat, 6W
(Mean ± SE, n=4)

Figure 33



Mean ± SE
*P<0.05 vs TPTX

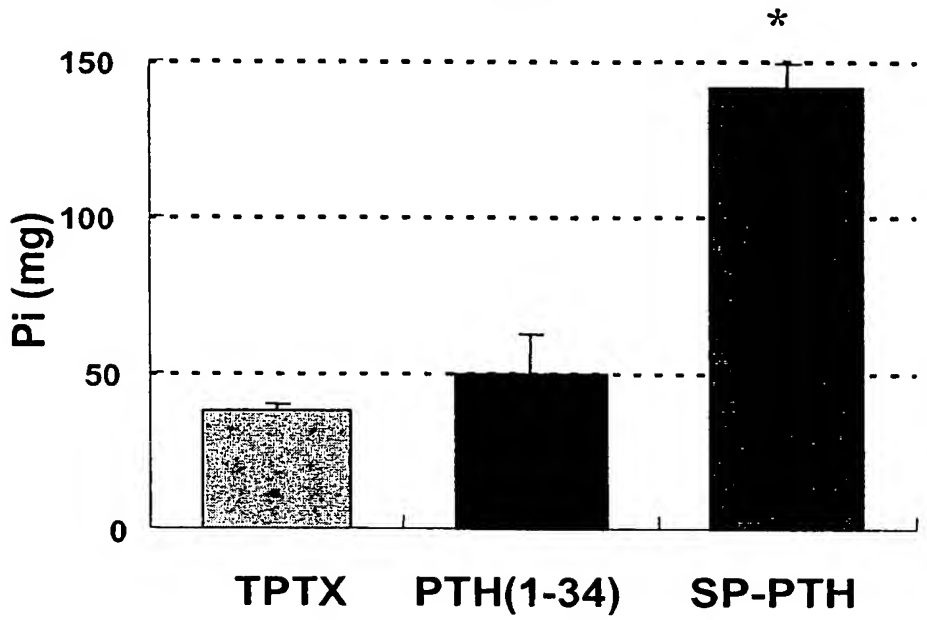
Figure 34



Animal : Crj:CD(SD) rat, 6W
(Mean ± SE, n=4)

Sham
Vehicle
PTH(1-34) 1.25 nmol/kg
SP-PTH 1.25 nmol/kg

Figure 35



Mean ± SE

*P<0.05 vs TPTX

Figure 36

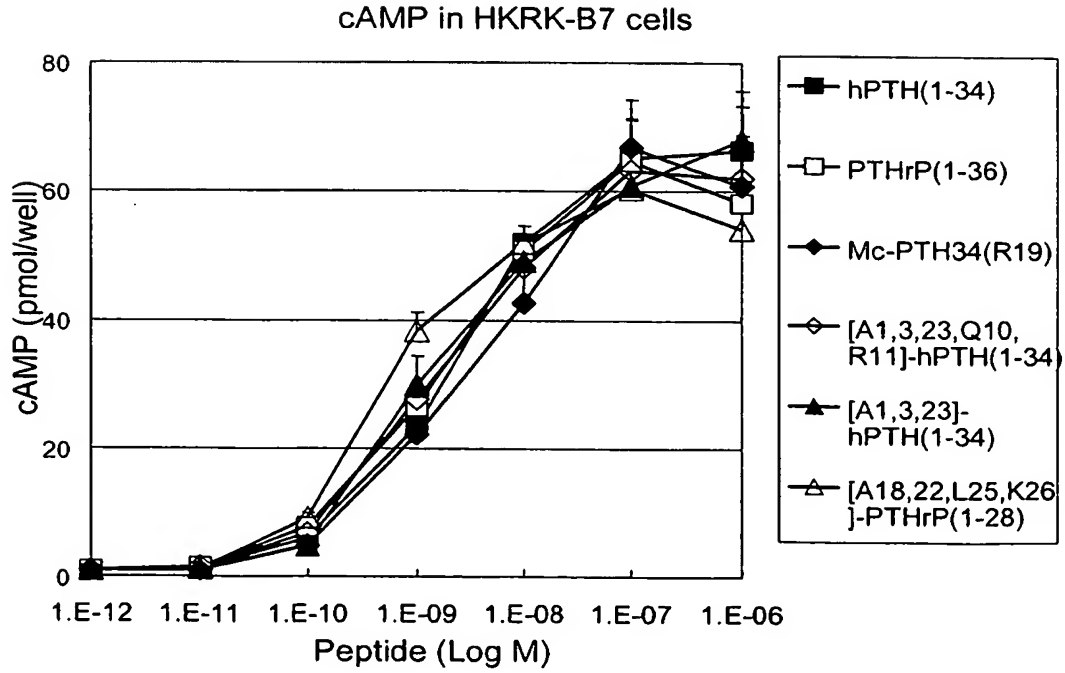


Figure 37